

# **Determination of the *in vitro* and *in vivo* Oral Drug Delivery Capabilities of Complexation Hydrogels**

A Thesis

Submitted to the Faculty

Of

Drexel University

By

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In partial fulfillment of the

Requirements for the degree

Of

Doctor of Philosophy

December 2006



## **Acknowledgements**

As I sit to write this last section, I am amazed that it could easily be the longest in this thesis. Six years, 1 child, 3 promotions, 2 companies and 4 supervisors later, it occurs to me that my time at Drexel has been a positive constant in my life for quite a while now. As a result, please bear with me as I could not think of cutting this portion short in fairness to all those that have played a part.

First and foremost, thanks and love to Lisa Gail for representing constant love and support for the last 15 years of my life. I love you. Thanks to Sammy for the smiles and understanding of what it means when ‘daddy has to work’ and for giving me the good reasons to stop when it is time to play.

Thanks to Tony Lowman for his patience, understanding and guidance, not just as an advisor but as a friend. He always knew what I needed and when. He understood the tension between work and school and never faltered, even when I really, really wanted him to. Thanks Boss.

I want to thank my family for the inspiration and belief. I owe so much to Gail, Frank, Dottie, Auntie and UG, Ma, Ryan, Mike, Rayne, Marissa, Carolann, Grant, Terry and Mac but all I can say is ‘thank you’. For Dad, Lauren, Matt and Grant II, I miss you every single day but I know you are close by.

Thank you to my committee members Dr. Palmese, Dr. Wrenn, and, especially, Hugh and John for all your guidance and support. Taking this on for me was never your job and it has meant a great deal.

I want to give a special thanks to Tony ‘LT’ Tuesca. One of the most satisfying aspects of the last few years has been watching you grow from student to

teacher. I hope, and expect, to be working for you one day. I also want to thank all my other Lowman lab colleagues, especially Meredith, Jon, Bob, Tom, Becky, Karri, Noelle, Jen and Jason for all the help and support. All the best to each of you and please feel free to call on me whenever you feel I can be of service.

Lastly, but not least of all, I would like to thank J&J and my J&J colleagues for the financial, emotional, technical and spiritual support over the last six years. To Ed, Bill, Dave, Todd, Michael, Jay and John, thanks for your leadership and for allowing and encouraging me to keep school as a priority. For Sue Dillon, thanks for the kind support, mentorship and for serving as a role model, especially when you weren't looking.

Thanks so much to Cindy Duchala, Gordon Powers, Greg Bannish, Mike Ryczyn, Debra Kwok, Stacy Hudgins, Eva Emmell, Peter Bugelski and George Tracy, Steve DiDomenico, Susan Tam and their staffs in the cell culture research, vivarium, assay and hot development labs at Centocor R&D for all your help and support. This goal would simply have never been possible without all of you.

Thanks to all of my staff, past and present for supporting me all these years. I know that each of you has gone without at times so that I could succeed. Thank you 100 times and, I know, that may be leaving some of you out. A special thanks to Ramin, Rob, Dave and Carlyne, you each have played a bigger part than you know!

Lastly, thanks so much to Jeff Joesph and his gang at TJU for getting me over the top and helping me finish strong so strong.

I told you I could go on...but I will stop here. Thank You.

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**Abstract**

Determination of the *in vitro* and *in vivo* Oral Drug Delivery Capabilities of Complexation Hydrogels.

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The promise of oral delivery formulations of therapeutic protein and peptides is one of high hopes but also of disappointment as, to date, parenteral administration remains the standard of care. A good example of this is in the case of human insulin delivery for diabetes where disease control, reduction of degenerative side effects and increased patient compliance could all be gained in the most rapidly growing disease in our country. Hydrogel carrier systems have shown promise as a potential solution for this unmet medical need. In particular a novel pH-sensitive poly[methacrylic acid-grafted-poly(ethylene glycol)] hydrogel system has shown promise by displaying enhanced insulin delivery in *in situ* animal models.

In this work, a set of detailed *in vitro* and *in vivo* experiments were used to further qualify the insulin delivery capabilities of this novel system. The ability of the system to protect entrapped insulin in the upper gastrointestinal tract was confirmed via dissolution studies and the ability of the polymer system to enhance transport across the intestinal epithelium was confirmed using the Caco-2 *in vitro* model of intestinal transport. Lastly, the absolute oral bioavailability of insulin delivered via the hydrogel system was determined in rats and dogs. The application of pharmacokinetic models to the animal data shows the greatest gains on improving this delivery system can be made by optimizing the ability of the hydrogel to enhance protein transport across the gut wall.



## CHAPTER 1: INTRODUCTION

The interest, demand and market potential for advanced drug delivery for hormones, antibodies and other protein drugs has been well documented [1]. Of particular interest is the ability to deliver proteins orally. Ideally, oral protein delivery has the potential to increase patient compliance, lower medical costs and provide superior pharmacokinetic profiles [2]. The primary barrier to effective oral protein delivery is the ability to obtain adequate bioavailability due to drug degradation by proteolytic enzymes and poor transport as it passes through the gastrointestinal tract.

One promising approach to oral protein delivery is the use of hydrogel technology, which has been studied extensively over the last 2 decades [3-9]. Of particular interest are pH-sensitive complexation hydrogel systems, which have demonstrated significant promise in oral delivery, especially in the specific application of oral insulin delivery [10-13]. The poly(methacrylic acid-g-ethylene glycol) system developed through this work has demonstrated the ability to enhance insulin bioavailability in a closed loop *in vivo* model compared to insulin alone [12].

In an effort to better understand the capabilities of protein delivery systems, and of the complexation hydrogel system in particular, quantitative analysis of drug absorption, bioavailability, toxicity and the pharmacokinetic and pharmacodynamic properties are required.

*In vitro* cell culture models are commonly used in the study of drug absorption mechanisms [14]. The Caco-2 model, a human intestinal epithelial cell line, is thought to satisfy the need for an oral absorption model that predicts *in vivo* situations



in a fast and efficient manner [15]. These *in vitro* tools are very amendable to the testing of drug candidates in high numbers but the models are fairly idealized and are most often used for initial drug transport estimation via membrane permeability studies. To obtain data that comes closest to predicting outcomes in humans, animal models of absorption, distribution, metabolism and excretion (ADME) are still essential [16].

This research focuses on presenting a detailed *in vitro* and *in vivo* analysis of the oral drug delivery capabilities of a novel complexation hydrogel system using human insulin as the test article and target protein. This type of analysis is critical in assessing the suitability of any drug delivery system for testing in humans. Hydrogel preparations loaded with insulin were evaluated in membrane permeability studies using a Caco-2 cell monolayer model to assess the *in vitro* transport enhancing effects. The pharmacokinetics and absolute bioavailability of this same system was then studied in rats and dogs to provide an estimation of key ADME parameters.

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## **CHAPTER 2: BACKGROUND**

### **2.1 Protein and peptide therapeutics**

Protein and peptide therapeutics can be defined and differentiated from small molecule therapeutics in several important ways. Besides differences in size, i.e. molecular weight, and complexity such as proteins with tertiary and quaternary structures, the complexities of manufacture, risks of contamination, purification and cost of goods are all distinguishing factors. Conversely, it is more straightforward to define small molecule therapeutics and non-peptide molecules that are ‘drug-like’ as per the ‘Pfizer Rule of 5’[1]. This model suggests that a molecule is drug-like if it has the following properties: molecular weight under 500; fewer than 5 hydrogen bond donors; a logP of less than 5 and fewer than 10 hydrogen bond acceptors. Important subclasses of protein and peptide therapeutics include human insulins and analogues, growth hormone, interferons, monoclonal antibodies, antimicrobial peptides, tissue plasminogen activators, fusion proteins and hematopoietic factors [2]. This chapter will present a comprehensive review of the utility, promise, challenges and economics of this important class of medicines.

#### **2.1.1 Unmet medical need and commercial opportunity**

Estimates vary by source but there is consensus that the worldwide market for protein and peptide therapeutics will reach \$100 billion by 2008 [3, 4]. This is due, in part, to the dramatically increasing success rates for developing biotech drugs. For example, the success rates for rDNA therapeutics entering clinical study between 1990 and 1997 was 35%, which represents a 35% increase over the success rate for

analogous drugs entering study between 1980 and 1989 [5]. In fact, the success rates for the development of biotech drugs exceeds those of small molecules according to public information available through the US Food and Drug Administration (FDA).

Driving this expanding market is the tremendous volume of unmet medical need in serious disease areas such as; arthritis, inflammation and immune disorders, central nervous system, diabetes and endocrinology, genetic disorder, hematology, infections diseases and oncology [3]. Many feel that the future treatments of many diseases in these serious therapeutic disciplines lies mostly with biological medicines. The vision is compelling with an example being the promise of cancer treatments without cytotoxic drugs. This vision is further supported by the fact that small molecule chemistry is reaching the limitations of diversity and novelty. Clearly, the promise of improved treatment options for serious diseases as well as the continually growing business rationale will continue to drive the future development of biological medicines.

While protein and peptide therapeutics can be classified via several taxonomic approaches, the following classes will be described further in this work; antibody therapies, vaccines and hormone therapy. In addition, the specific case of diabetes therapy will be detailed.

### **2.1.2 Antibody therapies**

Monoclonal antibodies (mAbs) are defined as immunoglobulin (Ig) molecules that possess the same structure, as opposed to polyclonal antibodies, which are the typical immune response to antigenic challenge. Both types of antibodies target

foreign substances and can be used to effect a specific response to an immune-mediated disease process. Monoclonal antibodies are produced by the fusion of an individual B-lymphocyte with a cancer cell. The hybrid cell that results retains the antibody-producing capability of the B-lymphocyte with the virtual immortality of a cancer cell[6].

The first monoclonal antibody therapy, Muroonab –CD3 (a mouse IgG), was approved for use in 1986 for acute allograft rejection in transplant patients. Since then, more than 15 other monoclonal antibodies have entered the market for treatments from cancer to rheumatoid arthritis. While these therapies carry their own set of side effects and long-term adverse event data does not yet exist, they are considered very safe when compared with other therapy options used in diseases such as cancer and are often the best lines of defense[7]. That said, the promise of these medicines remains critically viewed due to the high costs of mAb therapies, which can exceed \$20,000 per year for the treatment of rheumatoid arthritis[8].

### **2.1.3 Vaccines**

Continuing in the theme of immunology, vaccines represent another important class of protein therapeutics. The purpose of vaccines is active immunization, which consists of the administration of antigen to a host to induce the formation of antibodies and cell-mediated immunity against the administered antigen[9]. While there are more than 18 vaccines in current use against infectious diseases, the limitation of active immunization is that the host already has been infected and has the antigen, thus vaccination with more antigen will not treat active disease. That

said, the importance of vaccines, prophylactically, in healthcare cannot be underestimated and the control of vaccine-preventable diseases in preschool children is sub-optimal, even in developed nations[10].

#### **2.1.4 Hormone therapy**

Hormonal signals integrate and coordinate the metabolic activities of different tissues and optimize the allocation of fuels and precursors to each organ [11]. Naturally occurring hormones are produced by endocrine glands such as the thyroid, adrenal, ovary, testis, pituitary, pancreas and parathyroid glands. Often, hormone replacement therapies, using natural extracts or synthetic equivalents, are necessary when an organ stops producing adequate amounts of hormone, as is the case of insulin in diabetes. There are so many hormone replacement therapies on the market, the drugs are more appropriately described in detail when associated with specific diseases and deficiencies. For example, there are more than 12 thyroid and antithyroid agents available on the market [12].

#### **2.1.5 Diabetes therapy**

According to the US Centers for Disease Control and prevention, the prevalence of diabetes in the United States in 2005 was 20.8 million people or 7% of the population. In 2002, diabetes was the sixth leading cause of death and these estimates probably represent under-reporting as death certificates may reflect other conditions that actually have diabetes as the underlying cause[13]. When broken down by age and ethnicity, the numbers can be even more startling. It is estimated

that 9.6% of people over 20 have diabetes and that ~21% of people over 60 are afflicted with the disease. Non-hispanic whites have the lowest adult incidence at just under 9% and non-hispanic blacks are at the high end with an incidence of 13.3%. Even more alarming are the rates of occurrence of type II diabetes in children, an issue that is closely linked with the obesity crisis [14]. These sobering statistics clearly reflect that diabetes has reached an epidemic scale in the US and the financial and personal costs are staggering. The American Diabetes Association, ADA, estimated the total costs for diabetes were \$98.2 billion in 1997 [15].

Nomenclature systems vary but there are currently four distinct types of diabetes mellitus (DM). Type I DM is defined by selective pancreatic  $\beta$  cell destruction and severe or complete insulin deficiency. Type I disease can be further characterized by cause as idiopathic or immune and insulin administration is mandatory for these patients [16]. Tissue resistance to insulin and relative deficiency in insulin secretion characterizes Type II Diabetes Mellitus. Insulin is not essential for approximately 70% of Type II diabetics but this can change over time and close glucose and side effect monitoring is essential. Type III DM refers to varied other causes of elevated glucose such as those seen in the absence of pancreatic disease, such as can be seen as a side effect to certain drug therapies. Lastly, gestational diabetes is referred to as Type IV diabetes mellitus in this system of nomenclature. Gestational diabetes is diagnosed in approximately 4% of pregnancies in the United States.

Although the specific treatments can vary greatly by subtype of disease, all diabetes treatments have the same goal; to adequately regulate blood glucose in order



to prevent the primary and secondary effects of hyperglycemia [17]. As previously stated, in Type I diabetes mellitus, this can only be accomplished by the administration of insulin. The latest thinking in the treatment of Type II diabetes includes monotherapy using an oral hypoglycemic agent from one of five drug classes: sulfonylureas, meglitinides, thiazolidinediones, biguanides and  $\alpha$ -glucosidase inhibitors [18]. However, studies have shown that after 3 years of treatment, more than half these patients will require additional agents for adequate control and most type II patients may eventually also require insulin [19]. Treatment in gestational diabetes (GDM) may consist of dietary control, the administration of insulin or oral hypoglycemic agents. The oral hypoglycemic agents are used with care however as studies have shown increased incidence of pre-eclampsia in patients treated with metformin when compared to those treated with insulin [20]. Type III diabetes is, by nature, tied to specific etiologies and is treated accordingly and as needed.

## **2.2 Drug Evaluation**

Despite the startling technological advances in genetics and biotechnology over the last decade, the pharmaceutical and biotechnology industries struggle to improve their success rates. Between 1990 and 2000, the number of new drugs launched annually remained essentially constant while the associated research and development costs rose by almost 250% [21]. Most failures, 43%, in clinical phases I-III, were due to insufficient efficacy. The next leading cause was toxicity [22]. Furthermore, 40% of drugs never make it to clinical development as they fail in pre-clinical development due to issues with absorption, distribution, metabolism and

excretion (ADME) [23]. As the costs of drug development skyrocket at the clinical development stages, the importance of drug evaluation at the late pre-clinical stages cannot be overstated. Studying the pharmacokinetic and pharmacodynamic profile of drug candidates early in high quality bioavailability, toxicity and ADME studies is essential to improving the outcomes and safety of clinical trials and these studies are appearing earlier and earlier in the drug development process.

### 2.2.1 Pharmacokinetics and ADME

Pharmacokinetic evaluation involves the determination of four essential parameters: bioavailability, volume of distribution, half-life and clearance. There can be confusion as to the differences between absorption and bioavailability. Absorption is best defined as the processes that are involved in transferring the drug from the site of administration into the venous blood. Bioavailability differs from absorption in that it includes the effects of hepatic metabolism [24]. Mathematically oral absolute bioavailability (F) can be defined as:

$$F = (AUC)_{Oral} / (AUC)_{i.v.} \quad (2.1)$$

with  $(AUC)_{Oral}$  defined as the area under the concentration versus time curve for the oral drug dose and  $(AUC)_{i.v.}$  is the area under the curve of the concentration versus time curve for the i.v. dose [2]. At equilibrium, the volume of distribution (V) can be determined from

$$A = V * C \quad (2.2)$$

where A is the amount of drug in the subject and C is the concentration of drug in the plasma. Immediately following intravenous administration, the volume of drug in the body is equal to the administered dose so the volume of distribution can also be obtained from the following relationship:

$$V = (i.v. \text{ dose}) / (AUC)_k \quad (2.3)$$

where  $k$  is the first order elimination rate constant. This relationship also assumes the drug follows single compartment pharmacokinetics. Clearance (CL) measures the ability of a body to eliminate a drug and is expressed in the units of flow, volume per unit time. The derivation begins with a model of drug elimination by a single organ, which is defined as the extraction ratio (ER):

$$ER = (C_A - C_V) / C_A \quad (2.4)$$

Where  $C_A$  is the drug concentration entering arterial blood and  $C_V$  is the concentration leaving the organ via venous blood. The clearance by an organ  $CL_R$  can then be determined by:

$$CL_R = Q_R * ER \quad (2.5)$$

where  $Q_R$  is the blood flow to the organ. Lastly, half-life ( $t_{1/2}$ ) of a drug depends on the volume of distribution and the clearance:

$$t_{1/2} = V/CL. \quad (2.6)$$

### 2.2.2 Pharmacodynamics

Pharmacokinetics (PK) describes what the body does to a drug whereas pharmacodynamics (PD) describes what the drug does to the body, hence pharmacodynamics relates a measured response to the pharmacokinetics of a given drug in a given model. The ultimate goal in understanding the PD for a given system is to be able to design an optimized dosage regimen that maximizes the desired biological effect obtained by a drug at a given dose and that simultaneously minimizes the chance of adverse reactions. This is referred to as the therapeutic window. As PD is the measured effects that a drug has on an organism, early efforts utilize animal models. It is typical to progress testing, starting with rodent species, up through higher mammals to primates and eventually humans. In addition, computational models of pharmacodynamics have been developed.

Gobburu et al have presented some advanced pharmacodynamic mathematical models and have applied these constructs to the quantification of the indirect pharmacological effects of corticosteroid, diuretic, growth hormone, rh-erythropoietin and insulin models [25]. The model applied to insulin kinetics was described by the authors as Type IV and is shown in equation 2.7 below:

$$\frac{dR}{dt} = k_{in} - k_{out} \cdot \left( 1 + \frac{S_{max} \cdot C^\gamma_P}{SC^\gamma_{50} + C^\gamma_P} \right) \cdot R \quad (2.7)$$

where  $R$  is the response,  $k_{in}$  the formation rate of the measured response,  $k_{out}$  is the first order elimination constant,  $\gamma$  is the sigmoidicity,  $S_{max}$  is the maximum fraction of inhibition and  $C_p$  were the concentrations after intravenous bolus dose or intravenous infusion as shown in equations 2.8 and 2.9 respectively.

$$C_p = \frac{D \cdot 1000}{V} \cdot e^{-k_{el} \cdot t} \quad (2.8)$$

$$C_p = \frac{\frac{D}{T} \cdot 1000}{V \cdot k_{el}} \cdot (e^{-k_{el} \cdot t} - 1) \cdot e^{-k_{el} \cdot t} \quad (2.9)$$

Here  $k_{el}$  is the elimination rate constant,  $V$  is the volume of distribution and  $D$  is the dose. Using this model, the pharmacodynamics of insulin action were compared between the subcutaneous, intraperitoneal and steady infusion delivery routes/approaches. The model did approximate experimental data reasonably and the importance of dosing in causing indirect pharmacological effects was confirmed.

Experimentally, early stage pharmacodynamic studies typically utilize rodent and other lower mammal models. While a detailed treatment is beyond the scope of this dissertation, an excellent overview of animal models of diabetes has been provides by Sima and Shafrir [26]. In their text, detailed descriptions of more than 17 animal models of diabetes are presented and well as the characteristics, history and example applications of each.

Lastly it should be noted that PK and PD data are often presented concurrently and interchangeably. This oversight is not surprising given the complex

interrelationships of the two disciplines. The differences of focus and experimental approaches and interpretations are real and complex, however and care must be taken in experimental design to ensure the correct interpretation of experimental results.

### **2.2.3 Toxicity**

More than 90% of drug withdrawals from the market are due to toxicity [22, 27]. This has been most often due to undetected/unreported liver and cardiovascular toxicities but could also be due to drug-drug interactions. Given the enormous cost of drug development as well as the potentially higher costs in liability to remove a drug from the market, toxicity testing is also appearing earlier in the drug discovery and development processes. The challenge is in determining the best definitions and testing approaches to determining toxicity.

Animal models remain the standard for experimental toxicity testing but how good are they? Some of the best data aggregation in the field of drug hepatotoxicity is the output of a joint initiative between representatives from the American Association for the study of Liver Diseases, the FDA, NIH and PhRMA, the pharmaceutical manufacturers association. This group presented a study of 214 human toxicities. It was found that 71% of these human toxicities could be associated with toxicities in animal models. Sixty-three percent of the toxicities were detectable in non-rodent models while 43% were detectable in rodents. In addition, only 36% were detectable in combinations of rodent and non-rodent species. While this sounds encouraging, it should be noted that the actual predictabilities are much lower when false positives and false negatives are considered. In fact, it was

determined that only 31/328 or 13% of actual hepatotoxicities were detected with this approach, hence all other forms are not included and could be missed [28]. The determination of toxicities associated with proteins and biologic therapies can be even more problematic as they are frequently immunogenic which can cloud the evaluation of toxicity [29]. In fact, the complexities in accurately classifying the potential toxicities of biologic drugs is the subject of open petition between the biotechnology industry and the FDA [30].

In addition to animal models, the utility and promise of *in silico* and *in vitro* toxicity models continues to grow and latest thinking points towards the need for a combined approach to yield optimal results [31].

#### **2.2.4 Toxicity Parameters**

A standard for toxicity evaluation in drugs is the LD50. LD refers to lethal dose and the LD50 value is defined as the dose at which death is expected in 50% of the treated animals [32]. The units of LD50 are typically reported in mass of substance per mass of body mass.

#### **2.2.5 *in vitro* ADME and toxicity screening**

As it is highly desirable to find alternatives to animal testing that have the potential for greater specificity at reduced cost, the field of *in vitro* ADME/Tox prediction continues to grow rapidly. Over the last three decades models using biological material ranging from continuous cell lines to complete embryos have been used [33]. In addition, toxicogenomics is a promising emerging field to supplement

the pathological, morphological, chemical and biochemical information typically collected in *in vitro* and *in vivo* studies [34, 35].

#### **2.2.6 *in silico* ADME and toxicity modeling**

In the case of *in silico* modeling, it appears that there are acceptable ‘predictive’ computational tools for well-understood mechanisms of toxicity such as mutagenicity or skin sensitivities but analogous models do not yet exist for more mechanistically complex situations such as acute and chronic organ toxicities [36, 37].

#### **2.2.7 *In vitro* models of intestinal absorption**

In an attempt to model *in vivo* situations in a quick and inexpensive fashion, *in vitro* models of intestinal transport and drug absorption have been developed. These models typically utilize purified human or mammalian cell lines to simulate whole tissues and organs. The cells can be developed rather quickly, grown in quantity as needed and offer a humane and cost effective alternative method to facilitate the rapid screening of many compounds. Early work focused on obtaining intestinal brush border membrane vesicles (BBMV) and similar biomembranes [38]. Using these membrane vesicles, it was soon shown that peptide transport was independent of sodium concentration and occurred by a ‘non-concentrative’ mechanism [39]. This study may have been one of the earliest studies demonstrating active transport. It was later shown that thyrotropin-releasing hormone (TRH) uptake in BBMVs was most likely also passive [40]. These types of studies were reasonable simulations of what



happens within the brush border but mammalian cells are far more complex than membrane vesicles and better models were needed.

Shortly after these early studies, an excellent review of the enhancement of intestinal absorption was published by van Hoggdalem et al.[41]. This work summarized the *in vivo* work to date and was followed by studies that began to quantify and qualify the transport parameters for small peptide drugs. At this time, *in situ* animal models were still in play. It was soon shown that passive and non-passive (paracellular and transcellular) transport are independent and can occur simultaneously [42]. One key early study on insulin absorption examined the effects of the variations of tissue morphology and enzymatic makeup through the length of the intestines. It was shown that the jejunum and the ileum were preferable sites for insulin absorption compared to the duodenum [43]. Similar conclusions were reached in analogous studies using oxytocin and vasopressin. It was theorized that the distal segments of the intestines have a higher level of paracellular permeability [44]. A later study by Ungell et al. characterized the permeability of 19 drugs across three regions of rat intestine. The results showed correlation between the drug molecular weight, hydrophilicity or hydrophobicity and absorption in these regions of the intestine in the hope of developing an accurate analytical tool to predict the absorptivity of modeled compounds [45]. Another important step in the development of *in vitro* transport models was the necessary mathematical treatment of the transport itself. Sinko et al proposed a reasonable model in 1993 that was based upon convection, permeability and chemical reaction [46].

One of the earliest *in vitro* models was the Caco-2 cell line. This cell line was isolated from a primary colonic tumor at the Memorial Sloan Kettering Cancer Center in New York City [47]. This cell line has become the standard in the modeling of *in vitro* intestinal transport and some of the specifics of this cell line are covered in the next section. Early work with the Caco-2 line evaluated the transport of bile acids. It was found that the transport of taurocholic acid was dependent on time in culture and that the apical (AP) to basolateral (BL) transport was 10 times greater than the BL-to-AP transport [48]. That same year, work was done to compare the Caco-2 cell line model with the *in vivo* closed loop approach and it was found that the cell monolayer provided a reasonable approximation of the expected permeability seen with the *in situ* system for a specific family of peptides [49].

Since then, the Caco-2 model has become the mainstay of *in vitro* intestinal transport science. Transport of many proteins and large molecules have been evaluated and optimized including erythropoietin, granulocyte stimulating factor (GSF), nanoparticles, various antibiotics, cosalene (a potent inhibitor of HIV replication), benzo[a]pyrene metabolites, leuprolide just to name a few examples that have been published in the public domain [50-54]. As this cell line enjoyed a brief period of favor in the pharmaceutical and biopharmaceutical industries in the late 1990s, it is a good assumption that the absorption properties of countless small and large molecules have been studied in this cell line. It should also be noted that many of these papers focused on the ability of vitamin B<sub>12</sub> to mediate and improve uptake and transport in this cell line. In 1996, Quaroni et al. provided an excellent review of the development, state, benefits and challenges of using cell culture models for drug

transport and metabolism studies [55]. They concluded that there were three well-established and relatively credible *in vitro* cell models, each having its own limitations. They were the IEC-type cell lines, the only ‘normal’ cell model, and the Caco-2 and the mucin producing HT-29 cells. The reader can consult this reference for a more detailed treatment of the technology and usage up to that time. Lastly, in the decade since the previously mentioned review it should be noted that the utility of many *in vitro* cell line models, while still in use, is still being questioned relative to *in vivo* data and approaches [56].

### **2.2.8 Caco-2 Model Development and Characteristics**

Bailey et al presented a detailed review of the utility of the Caco-2 cell line over a decade ago. Most interesting in this review were the correlations presented between permeability, bioavailability and partition coefficients for molecules with molecular weights under 600 [57]. While the review is excellent, the focus on small molecule drugs makes the findings only indirectly applicable to protein therapeutics. There have been attempts to mathematically model the Caco-2 system. In one attempt to model diffusion and partitioning, it was determined that the caco-2 model fit somewhere in between the artificial membrane models of 1-octanol and isopropylmyristate systems. Theoretically, this is clearly less than predictive with reasonable precision [58]. There are also many instances of comparison studies between the Caco-2 model and other intestinal models. Each appears to have its own merits and downsides. In one model, the IEC-18 cell line, a low-resistance rat model, was compared with Caco-2 cells and was shown to exhibit similar transport profiles

with lipophilic compounds but increased rates of transport of hydrophobic compounds transported paracellularly [59]. Articles of this type highlight the fact that the Caco-2 model is clearly the standard of choice.

As the standard, there has been a significant amount of work focused on optimizing this model. Approaches to optimization include: evolving the model to establish a mucous barrier, the utilization of simulated intestinal fluids to remove the non-ideality of HBSS transport buffers, using mixtures of analytes to obtain higher throughput, development of more rapid, reduced serum culture systems and the quantifying effects of using transport enhancers [60-65]. Each of these works offered its own improvements but each was also very specific to the culture conditions, the drug molecules studied and the transport mechanism, active or passive, studied. Lastly, a solid general overview of the effects of culture conditions including seeding density, number of passages, split ratios and time to confluence has been provided by Behrens et al [66]. It should be noted that the approach from this particular article were strongly incorporated into the Caco-2 culture methods utilized in this research.

### **2.3 Protein and peptide drug delivery**

Most protein and peptide drugs are labeled exclusively for parenteral administration. A parenteral drug formulation may be administered as a continuous intravenous (i.v.) infusion, injected intramuscularly (i.m.) or injected subcutaneously (s.c.) [2]. This is due to the significant barriers to oral administration such as liver metabolism, enzymatic degradation, very short drug half-lives and other pharmacokinetic and pharmacodynamic deficiencies.

### **2.3.1 Challenges of parenteral administration**

Despite being the standard of care for most protein therapeutics, parenteral drug delivery of novel biologics presents significant issues of cost, efficacy and compliance. The costs are staggering. It is estimated that, cost sharing for biologics, the patient co-pay, could reach up to \$1000 per dose as opposed to \$15-\$50 for other medications and the annual cost per patient for the biologic treatment of some chronic diseases could reach \$35,000 [67]. The products themselves can be extremely expensive to make and, as most parenteral drug administration must be performed by or under the supervision of a specially trained healthcare professional, the treatments have often been tied to medical office and clinic visits, which further add to the cost of these therapies [68]. Managed care has responded with specialty pharmacy networks and a strong push towards the availability of self-administered injectables.

While not nearly as expensive or as difficult to deliver, the purely human aspects are critically important variables in this equation when insulin delivery is considered. A recent study showed that up to 65% of patients with type 1 and type 2 diabetes, in clinical trials to assess self-delivery devices, were not confident in their ability to effectively self-manage their disease and as many as 25% of these folks described anxiety with respect to self-injection [69]. Lastly, there is a significant unmet medical need for novel drug delivery systems in pediatric care where lack of age appropriate drug formulations often leads to the off-label use of adult drug delivery approaches[70].

### 2.3.2 Pumps and implantable devices

As so much work has been done on optimizing alternatives for insulin therapy, the state of personal infusion pumps can be studied by focus on insulin delivery. Insulin Pumps have been in use to deliver insulin subcutaneously to diabetic patients since the 1970s and it is estimated that pump usage in developed countries such as the US may be used by up to 20% of type 1 diabetes patients [71]. The primary advantages realized to date with continuous subcutaneous insulin infusion (CSII) are improved nocturnal glycemic control, a minimization of the pre-breakfast blood glucose increase, a decrease in frequency of hypoglycemia and lower mean glucose concentrations [72]. The major challenges to the application of this approach to wider patient populations are suitability of specific diabetes disease states, the inability of certain patients to manage the technical details and complexities of CSII, the inability of pumps to utilize continuous glucose monitoring and high costs [73].

The physical implementation of the continuous infusion pump is relatively simple and consistent. The pump is a small, battery-powered, external device that administers insulin through a subcutaneous catheter. Insulin can be administered at a slow constant rate and bolus doses can be given before meals or at other times as desired. Although this simple design approach has become a successful standard of care for a large patient population, the unmet opportunity lies in the fact that this open-loop approach is sub-optimal. In an open-loop system, the device is externally regulated and controlled. There is no feedback mechanism that enables a response to a given stimuli. In the case of CSII, this means that a pump is simply programmed to deliver a certain volume of insulin over a certain period of time or as controlled bursts

of prescribed duration. The pump has no awareness of the blood glucose or insulin levels in a patient. The more idealized device is a closed loop system where the principles of CSII are combined with continuous glucose monitoring. In effect, this concept approaches the ideal of an artificial pancreas. There are two closed-loop approaches in development. There is the s.c.-s.c. approach, where subcutaneous glucose monitoring is combined with CSII as well as an implantable i.v.-i.p., where intravenous blood sampling is combined with intraperitoneal insulin delivery [74]. Although progress is being made, to date, the glucose monitoring capability remains the limiting factor in the development of such a system [75, 76]. The complexity and variability of glucose control in diabetes make the development of a generic delivery algorithm that is capable of providing adequate closed-loop control challenging [77]. Lastly, the use of pumps in treating diabetes is on the rise. A standard of care for diabetes in children has been published and guidelines to assist families and medical professionals in deciding which children could benefit from the use of insulin pumps are effectively in use [78].

Some of the latest breaking options in protein delivery have been enabled by the growth and realization in the fields of micro-and nano-technology. Miniscule electro-mechanical devices for drug delivery are becoming feasible. An example of the possible implementation of this type of system is also an idealized artificial pancreas where a small-scale biosensor and drug reservoir are combined and implanted to achieve a wireless and integrated system for drug release [79]. Systems under development include an implantable microchip device that contains an array of discrete mini reservoirs. Drug release from these reservoirs can be controlled via

telemetry. Promising pharmacokinetic results were obtained when this system was applied to the pulsatile release of leuprolide, a hormone currently marketed for the treatment of endometriosis and prostate cancer [80].

### **2.3.3 Drug-eluting stents**

Although most applications of drug-eluting stents (DES) have involved the use of small molecule therapeutics, this exciting example of the emerging field of combination products (drug/device) will most likely be utilized extensively in the targeted delivery of protein therapeutics. Metallic scaffolds, stents, have been used to prevent restenosis of blood vessels following balloon angioplasty. On their own, these stents helped manage balloon induced vascular damage but there were side effects such as damage to the blood vessel wall and inflammatory responses such as in-stent-restenosis [81]. As systemic drug treatments of these side effects were inadequate, using the stents themselves as a localized delivery platform solved the issue. The results have been startling and more than 2.5 million stents had been implanted as of February 2005 [82]. The realized benefits include higher drug levels in the local tissues, lower risk of systemic drug toxicities and controlled release of the therapeutic agent over a prolonged period of time. The wide variety of mechanisms of controlled drug release have been successfully employed in stents include diffusion, dissolution or degradation, ion exchange and prodrug based systems [83]. These different mechanisms are enabled by the application of specific coatings to the stents. In addition, stent manufacturers have taken steps to improve biocompatibility. These include heparin and phosphorylcholine coatings as well as anti-body eluting



stents. In one of the earliest applications of stents incorporating antibody delivery, it was shown that when monoclonal platelet GP IIb/IIIa receptor antibody AZ1 was eluted from a polymer-coated coronary artery stent in rabbits, platelet deposition was significantly reduced, cyclic blood flow variation was almost eliminated and mean blood flow and arterial patency rates were improved [84]. The success of these devices is startling and unprecedented. Currently, almost 90% of stent patients receive drug-eluting stents and the frequency of coronary bypass surgery has actually begun to decline for the first time since its introduction [85].

#### **2.3.4 Ocular delivery systems**

Since the early 1980's there has been significant interest in the study of protein and peptide drug delivery via the eye as an alternative to parenteral administration as it could be more cost effective, offer more rapid blood concentration, avoids the first pass hepatic effect as the compounds bypass portal circulation to the liver, offers precise dosing and simplicity of monitoring for side effects [86]. This promise has led to the study of the eye as a delivery route for many drugs such as ACTH, calcitonin,  $\beta$ -endorphin, glucagons, LHRH, oxytocin, somatostatin, vasopressin and others including insulin [2]. The most promising results typically were found in the presence of various absorption enhancers [87]. These enhancers are used to overcome barriers to the eye as a route for protein delivery. These barriers include tissue compartments and cross-ocular blood barriers as well as enzymes that are capable of degrading the drugs [88]. As the tissue of the discrete eye compartments is not homogeneous, specific pharmacokinetic models

have been proposed to aid in the understanding of ocular drug transport [89]. Similar to the case of intestinal absorption, the key to optimizing this route of delivery appears to lie with understanding and exploiting active transport receptors and processes [90].

### **2.3.5 Transdermal protein delivery**

Drug delivery via the skin holds similar promise to other non-parenteral routes: high patient compliance, low costs, pain-free, easy maintenance and observations as well as possible avoidance of first pass hepatic metabolism. That said, the realization of these possibilities has been slow to come primarily due to the core purpose of skin, to provide a low permeability protective coating for the body [91, 92]. There have been successes, however, especially with low molecular weight ( $MW < 500$  Da) lipophilic drugs. Transdermal estradiol patches are used by more than 1 million patients per year and are not associated with the liver damage that has been seen with oral formulations [93]. Similarly, the success of nicotine patches as an aid to the cessation of smoking is startling [94]. Despite these successes, the untapped potential of this route of drug administration is perceived to be very high. Current work focuses on various absorption enhancers, such as pressure waves, microneedles, sonophoresis, chemical enhancers, iontophoresis, electroporation and the use of lipid vesicles, any of which may hold the key to success [95].

It has been reported that a single pressure wave, generated by intense laser radiation, is sufficient to permeabilize the stratum corneum of the skin and enable the transport of macromolecules. Using multiple pressure waves, there has been success

in delivering therapeutic levels of insulin to rats [96]. Hollow microneedles are another approach to transport enhancement. Using arrays of needles to gently pierce the skin, diffusion rates of drugs can be improved. Current work includes the study of both solid and biodegradable microneedles [97, 98]. The utilization of ultrasound, sonophoresis, as an enhancer of skin permeability has had success in delivering insulin, erythropoietin and  $\gamma$ -interferon in *in vitro* studies in cadavers and *in vivo* animal models [99, 100]. Chemical penetration enhancers have shown utility and promise. These range from small molecule solvents such as water and urea to complex proteins, colloids and lipid vesicles [101, 102]. The key complexity with this approach appears to be a lack of generality due to the tight specificity of each drug to an optimal penetration enhancer [103]. Electroporation is a transitory structural perturbation of lipid bilayers by the application of pulses of high voltage electricity. This technique has been shown to increase the transport across the skin for some high molecular weight molecules [104]. Arguably, the most promising approach to date is iontophoretic drug delivery. This technique employs a small electrical potential to achieve a constant electrical current across an area of skin. The resulting amount of drug delivered is directly proportional to the quantity of charge passed, the duration of application of current and area of skin surface in contact with the active electrode compartment [105]. This approach has been investigated for utility in insulin delivery as well as for the delivery of many other therapeutic agents and Alza has recently received FDA approval to market IONSYS™, an iontophoretic device for fentanyl delivery [106, 107].

### **2.3.6 Inhalant and mucosal protein delivery**

As parenteral routes of delivery remain the standard of care due to the significant barriers to non-parenteral absorption which include enzymatic and physical obstacles, mucosal and pulmonary routes of delivery are also of interest [108, 109]. The lung is a somewhat idealized delivery target due to the wide array of receptors expressed by the pulmonary tissues [110]. Success has been had in delivering peptides through the pulmonary system as desmopressin and salmon calcitonin are currently commercially marketed as nasal spray formulations [2]. Pulmonary and specifically, nasal, drug delivery offers the potential benefits, and possible unwanted side effects, of local and systemic drug absorption. The obvious example of this are the cases of intranasal corticosteroids used in treating bronchial asthma and allergic rhinitis where the pharmacokinetics and pharmacodynamics have been documented [111, 112]. Pulmonary drug absorption varies based upon the physical properties of the drug as well as lung deposition which depends upon these physical properties as well as the state of the respiratory system [113].

The fact that the pulmonary system serves as an entrance point for so many diseases can also be applied to obtain advances in drug delivery technology. For example, induction of mucosal immunity may be an important tool in the prevention of serious public health challenges such as severe acute respiratory syndrome (SARS) [114]. The ability of drugs absorbed through the pulmonary system to elicit an immune response is similarly being studied in combined mucosal/parenteral delivery studies for vaccination against HIV and influenza [115-117]. The diversity of opportunity around pulmonary delivery can be accentuated by the consideration of

some recent studies which utilize aerosol therapies as delivery approaches for gene therapy in lung cancer [118].

Of specific interest to me is the study of nasal insulin formulations. The recent FDA approval of an inhaled human insulin product, Exubera™ (Pfizer, NY, NY), has brought the dream of delivery more complex proteins nasally to fruition. A wealth of knowledge was obtained during the development of this product that can be generally applied to other nasal delivery applications. For example, it was determined that tight control of drug particle size is essential for delivery with the optimum aerodynamic diameter for drug delivery via alveoli being 1-3  $\mu\text{m}$ . Larger particles tended to be trapped and deposited on the oropharynx and the upper airway before reaching the alveoli and smaller particles were often lost during exhalation [119]. The onset of action for inhaled insulin is significantly faster than that of subcutaneously administered human insulin but the duration of glucose lowering activity was similar [120]. Despite the optimism of this revolutionary product, questions remain about long-term safety, specifically with respect to insulin buildup within the lungs [121]. As promising as the nasal route appears, there is clear opportunity for better alternatives.

## **2.4 Oral protein delivery**

Holding the promise of decreased healthcare costs, increased patient compliance and superior safety and pharmacokinetic profiles, the interest and demand for oral peptide and protein delivery systems is very high [122]. The disadvantages and challenges of parenteral protein administration were outlined in detail earlier.

Unfortunately, parenteral administration remains the standard of care as the barriers to oral protein delivery remain more significant and insurmountable to those of conventional delivery. In order to have adequate bioavailability, a protein drug must pass safely through the low pH and protease-rich environment of the upper gastrointestinal tract, across the mucous lining of and then across/through the intestinal epithelial lining and into the blood stream. Once into the intestinal capillary system, the drug must still survive a pass through the liver in adequate quantity to ensure adequate drug bioavailability as shown in Figure 2.1. These significant barriers will be discussed in detail in the following sections.

#### **2.4.1 Chemical and Biochemical Barriers to Oral delivery**

After oral ingestion, protein drugs must pass through the stomach intact. The definition of intact for complex protein therapeutics includes all four levels of protein structure including complex conformations [11]. The gastric juice of the stomach contains a family of aspartic proteinases called pepsins, which are most active at pH 2.0 - 3.0. Pepsins function by breaking proteins down into polypeptides, which are then passed to the duodenum. As the polypeptides and remaining proteins pass into the duodenum, there is a sharp rise in pH, which could cause the protein to precipitate if it passes through its isoelectric point. In the duodenum, pancreatic enzymes, such as trypsin, chymotrypsin and carboxypeptidase A, attack the proteins [123]. As the proteins reach the brush border they are acted upon by exopeptidases as well as lysosomes and other organelles that can act as sites of protein degradation [124]. It is estimated that more than 90% of the proteolytic activity of the small intestine occurs

at the brush border membrane. The distribution of brush border enzymes is variable throughout the jejunum and small intestine and profiles of this distribution have been studied as potential variables to be exploited for protein delivery [125-127].

The mucous lining of the gastrointestinal tract also offers physical barriers to protein transport. As a visco-elastic gel, the mucous lining ensures that food contents/chyme move smoothly through the intestines to prevent damage caused by caustic agents. Not only does the mucous layer offer a mechanical obstacle as protein drugs can slide past and through without adequate contact with the intestinal brush border, the glycoprotein matrix of the gel itself can be a physical obstacle to protein transport and uptake [128].

The drug that passes through the system unabsorbed is lost in feces. When taken in total, the chemical and enzymatic barriers to protein drug delivery via the gastrointestinal tract remain almost as formidable as reported more than 15 years ago [129].

#### **2.4.2 Cellular Barriers to Oral delivery**

Once a protein therapeutic has been successful in surviving the biochemical environment of the stomach jejunum and brush border it may come into physical contact with the epithelial lining of the small intestine, which presents the next obstacle. A schematic and the possible routes of protein transport across an idealized epithelial cell monolayer is shown in Figure 2.2. The possibilities for successful transport are passive transcellular transport, passive paracellular transport, carrier-mediated uptake and carrier mediated efflux [130]. Transcellular transport requires

that proteins cross over through cell membranes passively or actively and paracellular transport covers the cases where molecules can cross the membrane by moving between the tight intracellular junctions. These tight junctions (TJ) completely circumnavigate each epithelial cell to form a continuous seal that separates the apical and basolateral membrane components.

Tight junctions serve the essential purposes of providing a barrier to random paracellular transport of components and ensure the availability of a selective intestinal transport pathway. Tight junctions are complex structures of strands that have been described as intramembranous, cylindrical, inverted micelles that appear to result from the linear fusion between the plasma membranes of epithelial cells [131, 132]. Groups of proteins, called claudins and occludins form the primary TJ seal [133]. These TJ structures appear to act with another cell-cell contact system called the adherin junction (AJ) and it is a complex of TJ and AJ components that appear to anchor cytoskeletal components [134]. While providing a region of opportunity for mechanisms of epithelial transport, this must be done very cautiously as specific health issues may arise from chronic disruption of the TJ barrier [135-140].

Transcellular transport involves intracellular transfer from the apical surface of a cell through the cytosol and across the basolateral cell surface. For lipophilic drugs, this process can happen passively via a series of partitioning events [2]. In the case of sugars and amino acids, a carrier-mediated process is required [141]. This carrier-mediated process can be somewhat enigmatic. If the native epithelial cell membrane already contains a receptor for the targeted drug, then the transporter can work as designed. However, this is seldom the case for most synthetic and many



naturally occurring proteins and peptides. Needless to say, the barriers to transcellular transport are most formidable with the apical membrane being the rate-limiting step [142]. The selectivity of these transporters is critical to normal cellular function as the selectivity of transporters effectively regulate the pharmacological, and, potentially, toxicological effects of drugs by limiting the distribution of these substances to tissues and the blood stream [143]. These processes are extremely complex and the current understanding and hypothesis of the transcellular components of insulin transport across intestinal epithelium will be covered in later sections.

#### **2.4.3 Hepatic Barriers to Oral delivery**

As a key mechanism of drug clearance, the hepatic system poses a quantifiable barrier to bioavailability as drugs entering the venous capillaries will pass through the liver prior to becoming systemically available via arterial blood [142]. The hepatic clearance has historically been predicted via *in vivo* methodologies in combination with *in vitro* studies using using hepatocytes, hepatic microsomes and/or liver slices [144-146]. In truth, there is little that could, or should, be done to decrease or eliminate hepatic clearance of drugs to increase bioavailability as the homeostasis of liver function must be preserved to ensure health and well being.

#### **2.4.4 Commercial Barriers**

Another important, if non-intuitive, aspect of the feasibility of oral protein drugs is the economical impact. The promise of oral delivery presents a dichotomy in

that it is unclear who, if anyone, would benefit most. As biotechnology drugs are so complex, the method of administration is part of the specific license to market and label approved by the FDA in the US and foreign regulatory agencies abroad. This is primarily due to the complexities of demonstrating bio-equivalence between various formulations of a complex biological product [147]. This raises the question as to whether the introduction of a new formulation/delivery methodology can actually extend the patent life of a drug and/or be a way to deter competition from biological generics or biosimilars [148, 149]. This may, in fact, be necessary to make non-parenteral routes of delivery a reality for complex biological products.

The challenge lies in the complexity of manufacturing these drugs and the resulting eventual costs to the healthcare systems and consumers. The list of challenges is amazing, as the more successful a product becomes, the tighter the bottleneck that appears on the manufacturing end and, eventually, the higher prices soar [150]. This sets up a critical dependency between drug delivery and pricing with bioavailability as the relating factor. For example, if a major price driver for a protein therapeutic is the cost of manufacture, is it economically realistic to choose to deliver the drug to the patient orally or transdermally when 90% of the actual drug may be lost via the barriers discussed earlier in this chapter? Even with the costs of setting up infusion clinics and other necessities of parenteral administration, direct injections may prove to be the most cost effective treatment options. The recent approval of inhalable insulin, EXUBERA™, should serve as the first test of this complex economic picture. Will it be a commercial and medical success?

## **2.5 Options for Improving Oral Delivery**

Despite the previously outlined challenges and barriers, there have been numerous innovative approaches taken to get around these obstacles and deliver on the promise of oral protein delivery. The following sections will detail many of these approaches with a specific bias towards the applications of these approaches to metabolic therapy and insulin delivery.

### **2.5.1 Chemical Modification**

Chemical modification refers to actions taken to chemically alter or modify the drug properties to improve oral bioavailability, PK and/or PD without any resultant changes in drug function. Given the assumption that idealized properties for intestinal absorption can be described, a roadmap for chemical modification can be applied [151-153]. These include inclusion of small molecule carrier solutions, optimization of size, charge and hydrophobicity, lipophilicity, physical optimization of protein to the requirements of a specific transporter, octanol:water partition coefficients, N- and C- terminal modifications, prodrug approaches, peptidomimetic approaches, co-administration and/or incorporation of peptidase inhibitors, partial unfolding and several other approaches [154, 155]. Generally, these techniques are more successful with peptides than complete proteins due to the differences in molecular complexity.

Examples of protein modification attempts can be found across a range of therapeutic proteins. Conjugation of proteins to peptides and other molecules is an area of specific interest and intense study. A polycarbophil-cysteine conjugate system was utilized along with glutathione, a permeation mediator, to demonstrate an

improvement in the oral bioavailability of low molecular weight heparin [156]. A glutathione-mediated mechanism of action for this system has been attributed to inhibition of the closing of tight junctions via the thiol groups of the polymer [157]. Thiol chemistry was similarly important to an approach that utilized a chitosan-pepstatin conjugate to target calcitonin delivery to the stomach [158]. Using another approach, studies have demonstrated that conjugation of lipidic amino acids to poorly absorbed drugs to increase in lipophilicity can increase uptake [159]. This approach has showed promise in the case of the enzymatically labile proteins luteinizing hormone releasing hormone and thyrotropin releasing hormone [160]. This concept of application of peptides to increase drug uptake has also been applied to the specific task of cell penetration and continues to be an area of optimism due to the distinct specificity that this approach can afford [161, 162]. Salmon calcitonin has been utilized as a model drug for lipidization studies as well and, in the case of mice and rats, improved pharmacokinetics were obtained as well as improvements in pharmacodynamic models of osteoporosis [163]. Lastly, not quite the same as chemical modification, the use of absorption enhancers is of interest [164, 165].

Insulin has been the target of chemical modification in attempts to improve oral bioavailability as well as to alter its pharmacokinetic and pharmacodynamic properties. Early studies focused on the use of physiologic surfactants, such as sodium dodecyl sulfate (SDS), to improve enteral absorption [166]. Cyclodextrins were also examined by the same group as chemo-protectants and absorption promoters with mixed results in rodent models [167]. Acylation is another area that has been studied as a possible area of opportunity to improve enteral absorption of

insulin. It was shown, however, that acylation did not improve intestinal absorption but that insulin uptake in the large intestine could be improved by increasing the number of caproic acid molecules attached to insulin [168, 169]. Lastly insulin analogues have been developed and are available as parenteral formulations to provide varying pharmacodynamic profiles, which can be of benefit to specific patient groups [170, 171].

### **2.5.2 Protease Inhibitors**

Another approach to improving the oral bioavailability of protein therapeutics is to protect the drug from proteolytic degradation by inhibiting or reducing the action of the various enzymes that are capable of deactivating the molecule. Qualitatively, the enzymatic barrier to drug absorption is defined by the structure of the protein which is being orally administered. Selection of inhibitors must then be very specific against the specific enzymatic barriers [172]. As this approach is not limited to attempts to enhance oral delivery, it has been studied extensively. Many molecules have been evaluated as potential absorption enhancers including nafamostat mesilate, bacitracin, soybean trypsin inhibitor, chymostatin, potato carboxy peptidase inhibitor, phosphoramidon, antipain, leupeptin, bestatin, foroxymithin, amastatin, aprotonin, nafamostat, Tos-Lys-chloromethylketone, Tos-Phe-chloromethylketone, 3,4-dichloroisocoumarin, trans-epoxysuccinyl-leucylamido (4-guanido) butane and diisopropyl fluorophosphate (DFP) [173].

Various methods of administration have been attempted and some possible excipients themselves have been evaluated for their inhibitory properties. In an attempt to inhibit trypsin, starch-g-poly(acrylic acid) copolymers and

starch/poly(acrylic acid) mixtures were evaluated using Carobopol® 934P as the reference polymer for  $\text{Ca}^{2+}$  and  $\text{Zn}^{2+}$  binding and enzyme inhibition was observed [174]. Chitosans have also been chemically modified to serve as enzyme inhibitors and have shown inhibitory effects against pancreatic serine proteases [175]. There are many other examples including combination studies using multiple inhibitors that date back to the early 1990s [176].

There has been a significant volume of work focused on improving insulin intestinal absorption and enteral bioavailability using protease inhibitors. Early studies using closed loop intestinal models in the large intestine demonstrated successful significant increases in hypoglycemic effect when insulin was co-administered with 20mM of Na-glycholate, chemostat mesilate and bacitracin [177]. The same study concluded that soybean trypsin inhibitor and aprotonin improved insulin absorption in the small intestine. In *in vitro* models, hyaluronidase has been used to actually temporarily diminish the mucous/glycocalyx layer to remove the physical and enzymatic barriers to intestinal transport of insulin [178]. Similarly, studies have shown that co-administration of the protease inhibitor, aprotonin, increased the pharmacodynamic effect even more [179]. Lastly, there has been effort applied to enzyme inhibitors to protective polymeric delivery systems. Conjugates of the mucoadhesive polymer sodium carboxymethylcellulose with the Bowman-Birk inhibitor (BBI) showed strong inhibitory activity against trypsin and chymotrypsin and polymer-elastinal conjugates demonstrated better inhibitory activity against elastase [180]. This study further accentuates the role of specificity in the success of these approaches.

### 2.5.3 Mechanistic enhancers

An alternative method to increase drug absorption is to design strategies that target specific mechanisms within the cellular and intracellular armor itself.

Previously mentioned examples of this approach applied to non-oral systems include the iontophoretic delivery approach for drug transport across the skin. In this case, an electrical current is used to temporarily and reversibly make the skin more permeable to therapeutic agents [105]. One analogous approach to the enhancement of oral drug delivery is to alter paracellular drug transport via manipulation of tight junctions.

A model of tight junction structure is presented in Figure 2.3. The tight junction is a multiple unit structure composed of a multiprotein complex consisting of transmembrane proteins; occluding and claudin and cytoplasmic plaque proteins; ZO-1, ZO-2, ZO-3, cingulin and 7H6 that are affiliated with an underlying apical actomyosin ring [137, 181]. *In vitro* and *in vivo* studies using Zonula Occludens toxin (Zot) and its biologically active fragment,  $\Delta G$  as absorption enhancers, have shown promise in the ability to transport paracellular markers and previously poorly absorbed drugs across the intestinal epithelium [137]. This approach has been utilized in combination with the enzymatic degradation inhibitors bestatin, captopril and leupeptin to demonstrate significant increases, 200%-5000%, in the oral bioavailability of radiolabeled cyclosporin, ritonavir, saquinavir and acyclovir when administered intraduodenally [182]. Similar studies have been done to demonstrate the ability of  $\Delta G$  to enhance the paracellular transport and oral absorption of mannitol [183, 184]. Similarly, the ability of Zot to reversibly open tight junctions has been studied in the application to bovine brain microvessels to enhance transport of

molecular weight markers and anti-cancer chemotherapeutic agents, such as [ $^3\text{H}$ ]doxorubicin, MTX and paclitaxel with some success [185, 186]. Zot has also been evaluated as a mucosal adjuvant to induce protective immune responses for the application of vaccine delivery [187]. Despite the promising results, there are concerns regarding the unintended negative consequences of tight junction function [188]. Most of these studies have tested cellular toxicity but the physiological effects of tight junction disruption in *in vivo* studies is seldom cited. As previously mentioned there are many mal-absorption illnesses associated with altered intestinal permeability and similar concerns have been raised for applications of drug delivery enhancement to airway epithelia. Targeting the occluding proteins directly has been studied as a potentially less toxic alternative [189]. Lastly, as described in section 2.4.2, a potentially more promising mechanistic approach is to target specific cell membrane carrier enzymes for active transcellular transport and several interesting *in vitro* screening approaches have been developed [190, 191].

#### **2.5.4 Site Specific Drug Delivery: Colonic, Lymphatic and Others**

In this section the concept of enhancing bioavailability by targeting therapeutics to specific organ and tissues via oral and other routes of administration are discussed. The colon is one such commonly targeted organ. Typically a carrier system, such as those that will be discussed in the next section, is employed to enable safe passage of the drug through the stomach and small intestine [192]. One reason to target a specific organ is to treat organ-specific diseases locally. One example is the design and use of polymer conjugates to bring colon cancer therapies directly to the



colon following oral administration [193]. Alternatively, the varying regions of the intestinal system may be targeted as absorption varies geographically throughout the tract [194, 195]. The differences may be attributable to differences in the distribution of cellular receptor sites, mucous thickness, differences in tissue permeability and mass transport challenges due to chyme and fecal matter [196]. Lastly, the slow moving environment of the colon may make it ideal for certain therapeutic situations [197].

Once to the colon, whether the drug was administered orally or rectally, a variety of techniques to enhance absorption and bioavailability have been employed. Absorption enhancers, protease inhibitors, enteric coatings and chemical modification have all been tried [198, 199]. In the case of insulin, it has been shown that absorption enhancers such as surfactants, bile acids, phospholipids, enamine derivatives and sodium salicylate derivatives are necessary to obtain reasonable bioavailability [200, 201]. Insulin absorption studies in the ascending colon of rats using a polymer, *N*-diethyl methyl chitosan (DEMC) as an absorption enhancer to loosen tight junctions, also demonstrated significant hypoglycemic effect [202].

Interestingly this approach to drug delivery contains a mixture of some of the older approaches to oral delivery, colonic targeting, with some of the newest as more advanced delivery mechanisms are becoming necessary in order to deliver more advanced biological products such as larger and more complex proteins, oligonucleotides and whole genes [203-205]. Exciting work is being done to target delivery of complex therapeutics to the liver. Methodologies include antisense and

ribozyme techniques, viral and non-viral vectors, prodrugs and carrier systems, such as liposomes, are all under investigation [206-208].

Another area of specificity is the targeting of drugs, especially highly lipophilic drugs, to the lymphatic system. The role of the lymphatic system in distributing large and small molecule drugs following parenteral or subcutaneous administration is well studied [209, 210]. In addition, the intestinal lymphatics can be utilized as a specialized transport and absorption pathway for highly lipophilic drugs offering the advantages of avoidance of the first pass hepatic effect as well as specific targeting of drugs to the lymphatics [211]. One application is to use glyceride prodrug formulations of oral anti-inflammatory agents to avoid/reduce gastrointestinal irritation. Another novel indication is the ability to target anti-infectives, immunomodulatory and anti-viral agents to the lymphatic system to achieve adequate oral pharmacokinetics and pharmacodynamics in the treatment of infectious immune diseases such as HIV [212].

### **2.5.5 Carrier Systems**

One of the most promising approaches to oral delivery of proteins is the use of carrier systems. Systems utilizing polymer carriers, such as hydrogels, liposomes, nano- and microparticles and erythrocytes are all under investigation but the overall concept is similar. The drug of interest is incorporated into or conjugated on to the carrier and administered orally. The carrier then serves one or more of the following functions: to protect the protein from enzymatic degradation, to entrap the protein until it has reached the organ or tissue of choice, enable controlled release kinetics or

to improve targeted uptake and/or transport once a target tissue is reached. The section that follows will provide a comprehensive review of carrier systems for oral protein delivery.

## **2.6 Polymeric Delivery Systems**

The concept of using a plastic as a carrier and/or depot for the delivery of drugs dates back to the early 1960s when silicone rubber was used as an implantable carrier for low molecular weight drugs and this approach continues to be an area of significant promise [213]. Much of the earliest work focused upon implantable systems, often referred to as depot systems, for prolonged sustained release. Many of these early systems had the drawback of poorly controlled release and needed removal once therapy was complete. Various bioabsorbable polymers made of hydrogels, copolymers of polylactic and polyglycolic acids, polylactic acid, poly(orthoesters), polyanhydrides, poly(E-caprolactone) and polyurethanes were then developed and studied against a wide range of drugs [214]. By the mid 1980s, polymeric systems were being evaluated as carriers for oral drug delivery. These systems were based upon osmotic pumps, polymer-matrix slow release formulations and/or gastrointestinal bioadhesion [215, 216]. Since then, interest in the technology has blossomed, as have advances in the field of polymeric drug delivery. The sections that follow will detail the history and current state of polymeric drug delivery with a strong focus on oral protein delivery.

### 2.6.1 Hydrogel Delivery

A hydrogel can be generically defined as a colloidal gel in which water is the dispersion medium. These polymers mimic biological tissues and were first evaluated for use in ophthalmic products in the mid-1950s [217]. By the early 1990's hydrogels were being studied as potential enablers of oral protein delivery and the characterization of the complexities of the relationship between hydrogel structure and performance was underway. The first studies on the possible use of hydrogels for site specific oral protein delivery utilized novel biodegradable N-substituted (meth)acrylamide, N-tert-butylacrylamide and 4,4'-di(methacryloylamino)azobenzene gels. The gels were characterized by equilibrium swelling, elasticity in compression at varying pH and insulin permeability at varying pH and the degree of biodegradation was related to the degree of swelling [218, 219]. Further characterization determined that the polymer molecular weight and viscoelastic behavior of the gel has significant effects on the mechanisms of drug release with the lower molecular weight polymers displaying relatively equal rates of swelling and dissolution which resulted in a constant release rate of the drug etofylline [220].

As hydrogel characterization evolved, standard parameters were defined and utilized. Figure 2.4 shows an idealized hydrogel along with key parameters. In this model,  $Q$  is the volume degree of swelling,  $M_c$  is the molecular weight between crosslinks,  $\xi$  is the mesh size in Å,  $d_h$  is the hydrodynamic radius of a drug [221, 222]. These studies suggested that the transport of small molecular weight drugs from hydrogels is more impacted by the equilibrium-swelling ratio than by the degree of polymer crosslinking. This work built upon previously developed mathematical

models of drug diffusion and release from a dissolving polymer network [223, 224]. Soon, the concepts of bioadhesion and pH responsiveness were incorporated to facilitate delivery that was better controlled and more site specific [225, 226]. The approach of a mucoadhesive, pH-sensitive hydrogel system was then applied to the nasal delivery of budesonide, a seasonal rhinitis drug with low oral bioavailability [227]. The specific system was based upon novel copolymers of polymethacrylic acid and polyethylene glycol P(MAA-g-EG). This system will be covered in detail in an upcoming section, as it is the core focus of this dissertation.

There are many other hydrogel systems that have been utilized in oral drug delivery, as the applications seem more endless than the list of drugs that these systems attempt to deliver. The pharmacokinetics of chloroquine, a drug used to treat malaria, were assessed following administration via amidated pectin chloroquine beads suggested drug release in the duodenum, jejunum or ileum [228]. The release of antifungal agents, such as chlorhexidine, from chitosan hydrogels has also been evaluated [229]. The capabilities and behaviors of hydrogels composed of phospholipid polymers have been well studied. The swelling and release of insulin and cytochrome c were studied in hydrogels made from 2-methacryloyloxyethyl phosphorylcholine copolymers and the insulin transport was found to be dependent upon the swelling and dissolution of the polymer chains [230, 231]. Pluronic gels have been evaluated for the capability to deliver Vitamin B<sub>12</sub> nasally and polyvinyl alcohol hydrogels demonstrated the ability to deliver tylosin orally but not oxytetracycline [232, 233].

Hydrogel delivery systems comprised of crosslinked alginate/N,O-carboxymethyl chitosan have been developed and evaluated as a pH-responsive system, as have hydrogels utilizing natural polysaccharides and hydrogels prepared from copolymers of 2-hydroxyethyl methacrylate and methacrylic acid and hydrogels based on bovine serum albumin [234-237]. The latter system, like the P(MAA-g-EG) systems to be discussed later, were evaluated as a potential oral delivery system for insulin.

Hydrogel devices such as one based upon bonded layers of a PMAA hydrogel and a poly(hydroxyethyl methacrylate) (PHEMA) hydrogel have been developed in order to provide mucoadhesion as well as unidirectional release [238]. Lastly, N-carboxymethylchitosan-based hydrogels have been evaluated for the ability to provide extended release of prednisolone following oral administration [239].

While all these applications have been developed there has been substantial work to further understand, clarify and optimize the mechanisms of action of hydrogels on the bench, *in vitro* and *in vivo*. Of specific importance are GI transit times. The ability of a hydrogel to remain present, and in contact, with certain tissues and organs can be valuable in establishing favorable pharmacokinetic profiles. In one study, the gastric transit and emptying times of super-porous hydrogels (SPHs) made from Ac-Di-Sol((R)) (croscarmellose sodium) were evaluated in dogs. It was determined that, in fasted animals, the hydrogels remained in the stomach 2-3 hours. When the animals were fed, the SPH remained in the stomach more than 24 hours [240]. A similar study in man of SPH-composite hydrogels delivered in enteric-coated gelatin capsules demonstrated a stomach retention time of 75-150 minutes and that the polymers attached to the small intestine for 45-60 minutes [241]. In an

attempt to manipulate the mass transfer of the hydrogels through the GI tract and optimize protection from proteolytic enzymes, mucoadhesion strategies and composites of hydrogels with protective agents have also been studied [242, 243].

Lastly, it should be noted that pH-responsive hydrogels are only one type of environmentally responsive gel. Hydrogels have been engineered that are temperature sensitive, glucose sensitive, electrical-signal sensitive and light sensitive just to name a few and an excellent review of these other approaches is available [244].

### **2.6.2 Micro- and Nanoparticle Delivery**

Colloidal oral delivery systems, such as micro- and nanoparticles are intended to improve pharmacokinetic and pharmacodynamic properties of drugs via improved mass transport mechanisms. The very small particles are capable of delivering interesting diffusion profiles of their payloads, superior bioadhesion with the gastric mucosa and may, themselves, transfer into the blood stream, into cells or other site specific destinations [245-249].

Micro and nanoparticle systems have been developed from a wide range of chemical approaches with the solid-lipid nanoparticle being the most common theme [250]. Particles have been prepared from lectins and invasions, crosslinked polysaccharides, polysaccharide chitosan, graft copolymers such as P(MAA-g-EG) and poly(D,L-lactic-co-glycolic acid), mesoporous silicon as well as natural products such as human serum albumin just to name a few [227, 251-257]. The design optimization criteria of these systems are particle size, chemical structures, surface

characteristics, mucoadhesion and drug-specific loading and release characteristics [258-261].

There are many applications of these colloidal delivery systems. The promise of targeted therapies to specific organs, tissues and cells have made them of particular interest in oncology where systems have been developed and studied for the delivery of cytoprotectives, cytotoxics and antibody therapies [262-268]. With similar hopes, nano-and microparticle approaches have also been developed to deliver anti-infectives, antivirals, vaccines and antiproliferatives [269-275].

As with most other non-parenteral protein delivery approaches, there is significant study ongoing for the delivery of insulin with particulate systems. Studies using CaCO(3)-nanoparticles to deliver insulin transdermally in rats show promise as do studies applying insulin-loaded chitosan nanoparticles for nasal delivery [276, 277]. Of course, the oral approach is preferred and there have been many attempts to optimize particulate systems for this purpose. Early rat studies using insulin incorporated into isobutylcyanoacrylate nanoparticles showed that the insulin was released in the intestine but concluded that most of the protein was lost to proteolytic degradation in the small intestine [278]. Studies that followed have used a variety of nanoparticle systems including chitosan, poly(N-isopropylacrylamide) and poly(ethylene glycol) 400 dimethacrylate, polysaccharide chitosan (CS-NPs), polyethylenimine and dextran sulfate, poly(n-butyl cyanacrylate), poly-epsilon-caprolactate and Eudragis, polyethylene glycol (PEG)ylated trimethyl chitosan and gold to varying levels of success [279-284]. In the case of CS-NPs, oral bioavailability of almost 15% relative to sc was obtained in diabetic rats and



prolonged hypoglycemic effects in rats were observed with several other nanoparticle formulations [278, 285-288]. Clearly there is more work to be done as this approach is still in the pre-clinical stages.

Lastly, it should be noted that there has been little work done to date to objectively study the safety of these systems. Cytotoxicity assays can be idealized and the particles themselves must be studied as well as the particle-drug combination. Concerns have been raised regarding the long-term affects of these systems. Some of these concerns parallel those related generically to polymeric drug delivery systems while some are specific to these colloidal particulate approaches [289, 290]. One such reported safety concern, the aggregation and associated long-term effects in the digestive tract, is one potential driver for the next approach to be discussed, biodegradable polymer delivery systems.

### **2.6.3 Biodegradable Polymers**

Carrier systems based on biodegradable polymers are of interest as the retention time *in vivo* can be designed and optimized. Once the drug has been released, the polymers are broken down into inert components and eliminated from the body naturally. This approach eliminates the concerns of polymer buildup in the body that have been previously discussed and this approach is essential when polymer nanoparticles are taken up by cells, tissues and organs. Several of the carrier systems discussed previously are designed to be biodegradable [214, 252, 270, 275]. As is the case with most systems discussed so far, the polymer delivery vehicle and the target therapeutic must be specifically designed for each application.

One area of interest and application for biodegradable nanoparticles is in inflammatory bowel disease. The small size, under 200  $\mu\text{m}$ , enables the particles to avoid causing diarrhea symptoms and the particles have demonstrated the ability to be taken up into the macrophages and other immune-related cells that congregate at the sites of bowel inflammation [291]. For this purpose, Rolipram, an anti-inflammatory model drug, was administered to rats using poly(lactic-co-glycolic acid) nanoparticles demonstrated fewer adverse effects than solutions. A similar study in mice demonstrated reduced drug toxicity when Tacrolimus was administered via PLGA or pH-sensitive Eudragit nanoparticles versus oral or subcutaneous administration of the drug alone.

This ability to target macrophages has also been applied to cancer therapy, allergy therapy/oral vaccines and in immune mediated diseases such as rheumatoid arthritis. One set of experiments using PLGA nanoparticles to deliver paclitaxel in the HT-29 cancer cell line showed that the nanoparticles were taken up by the cells and that the cell mortality caused by the nanoparticle formulation was 13 times higher than that caused by drug alone [292]. PLGA particles have also been applied with some success in the oral treatment of Type I allergy [293]. Lastly, a nanoparticle system containing entrapped PEG-conjugated immunodominant peptides have shown promise in *in vitro* models of immune mediated and inflammatory disease [294].

#### **2.6.4 Liposomes**

Liposomes are amongst the oldest and most successful carrier systems for targeted drug delivery [295]. Liposomes are lipid bilayer vesicles prepared from mixtures of lipids that are excellent mimics of the naturally lipid bilayers of cells and

organelles [296]. This ‘likeness’ to cell membranes makes them very biocompatible and enables liposome formulations to cross cell membranes and target specific cells and tissues. Liposomes can be used to encapsulate and transport drugs of diverse nature including hydrophilic, lipophilic and amphiphilic molecules. This is because the drugs can be entrapped either within the membrane-enclosed aqueous compartment or bound by direct association with the lipid bilayer as shown in Figure 2.5 [297]. Early studies for oral targeted delivery of antigens to gut-associated lymphoid tissue demonstrated the rapid uptake of liposomes in the Peyer’s patches of the lower ileum in rats [298]. While cellular uptake can be direct and quick, subsequent rapid degradation of the liposome formulations *in vivo* can lead to short half-lives. This has led to the development of stealth liposomes, which are liposome formulations that are modified with polyethylene glycol (PEG)–lipid derivatives. The bulky PEG group prolongs the liposome life by inhibiting uptake into the reticuloendothelial system [299]. This approach has led to the successful approval and marketing of injectable liposome formulations in oncology, doxorubicin and daunorubicin and in anti-fungals, amphotericin [300].

Liposomes have been evaluated as carrier systems for insulin. Early studies demonstrated the ability of liposomal insulin to target hepatocytes when administered intravenously but the oral liposomal formulations resulted in an unacceptable amount of variability in the glycemic response [301]. To improve bioavailability, the coating strategy discussed above has been employed using PEG-2000 and mucin coatings. Aimed at prolonging intestinal transit times, the study showed that the mucin-coated liposomes had a longer gastric retention time but intestinal transport was unaffected.

However, the PEG-2000 coated liposomal formulation was retained in the small intestine much longer via interaction with the mucous layer [302]. Chitosan coating has also been used to improve the hypoglycemic efficiency of oral insulin delivered via liposomes in mice [303]. Similarly lectin-modified insulin liposomes have shown promise as a relative bioavailability of 9.12% when compared to subcutaneous insulin has been achieved [304]. While these results are promising, there is a significant gap that must be bridged as oral absolute bioavailability of 10% or greater is required for serious commercial interest.

### **2.6.5 Micelles**

Micelles are another class of nanoparticle structures that are of specific interest in advanced drug delivery. Like liposomes and lipid bilayers, micelles can be generically defined as amphipathic lipid aggregates that naturally form in water as shown in Figure 2.6 [11]. Micelle-like structures can be formed using block copolymers, which enable the utility of combining the desired aspects of more than one homopolymer [305]. Micelles can self-assemble from block copolymers, which have hydrophilic and hydrophobic segments as shown in Figure 6.

The small size, typically 10-100 nm, of micelles and the hydrophilic outer core offer the drug entrapment possibilities of liposomes but without significant risk of premature dissolution by the reticuloendothelial system (MPS). Further the in vivo stability of micelle-drug formulations is strongly related to the physical state of the core-forming polymer with micelles having a glass transition temperature ( $T_g$ ) of

greater than 37°C being considered very stable [306]. Drug can be released from micelles either through partitioning or via disintegration of the micelles. Preliminary studies confirmed the pH-responsiveness and the ability to release progesterone in an *in vitro* model [309]. Micelle systems based upon poly(hexyl-substituted lactides), poly(lactide)-poly(ethylene glycol), DEX-g-PEO-C<sub>16</sub> and self-assembling PEG-*p*(CL-co-TMC) copolymers have all been studied as potential carrier systems for hydrophobic drugs [310-313]. Lastly, there is little available information to support that much effort has been applied towards the delivery of insulin via micelles.

#### **2.6.6 Mucoadhesion**

As the above oral polymeric carrier systems have been discussed, it was apparent that many of the systems were designed to optimize the corresponding mucoadhesive properties. The purpose of mucoadhesion is twofold. First, it is assumed that mucoadhesive delivery systems can improve pharmacokinetics by ensuring intimate and prolonged contact with the designated tissues in the gastrointestinal system. Second, this prolonged contact should reduce the loss of drug via fecal elimination, as the carrier should stick to the gut walls once contact is made. The mass transfer situation of flow in the gastrointestinal tract has been modeled via the cases of peristaltic transport in a channel with a porous peripheral layer and as nonideal chemical reactors [314, 315]. The resulting mathematical models were complex as the movement of a core volume; a porous and static boundary layer, fluid trapping, axial mixing and reflux were all significant variables. A simplified model is

presented in Figure 2.7, which shows a laminar flow situation where the probability of wall contact of suspended particles is extremely complex.

Efforts to improve the mucoadhesive, or bioadhesive, properties of a given carrier system are, obviously, fairly specific to the polymer chemistry of the carrier system itself. Early work on controlled release tablets made of hydroxypropyl methylcellulose and poly (acrylic acid) demonstrated that the adhesion strength between the tablets and bovine gut mucosa was a monotonically increasing function of the poly (acrylic acid) content [316]. Soon after, the cationic charged chitosans were demonstrated to improve intestinal transport in *in situ* models due to its adhesive properties and the ability to bind to lectins on the walls of the GI tract [317]. It has often been the case that adhesion enhancers were combined in models with protease inhibitors in order to reduce the enzymatic degradation of the drug payload. This has led to the desire to develop a system that could inhibit a membrane-bound protease, even when the enzyme was protected by a mucous layer, by displaying a high binding affinity to bivalent cations [318].

More recent work still focuses on lectin binding as well as non-specific glyocalyx adhesion and virtually all polymeric carrier systems have mucoadhesion designed into the chemistry [242, 319]. One recent example in oral insulin delivery utilized a lectin wheat germ agglutinin (WGA) conjugated to alginate microparticle system in rats. It was determined that the WGA-alginate particles showed the most pronounced hypoglycemic effects but it was unclear if this effect was caused by prolonged residence time from mucoadhesion [320]. Hence, it is clear that theoretically and experimentally the concept of mucoadhesion is an important design

criteria for polymeric oral drug delivery systems. That said, the previously stated cautions regarding toxicity and potential buildup of polymer in the digestive tract long-term will need to be carefully evaluated in order to ensure regulatory approval of such systems.

### **2.6.7 Bioconjugation**

Bioconjugation is defined as the linking of two or more molecules to form a novel complex having the combined properties of the individual components [321]. This can be illustrated as the chemical modification of a protein with a polymeric carrier. Many examples of this chemical approach have been given in earlier sections such as the conjugation of a protease inhibitor to a polymeric carrier to protect a protein drug from proteolytic breakdown. In addition to these examples, bioconjugation itself can be a protein drug delivery strategy. By covalently bonding a polymer to a therapeutic protein, enzymatic degradation can be reduced as the polymer can act as a protective shield. Furthermore, polymer conjugation may improve pharmacokinetics by increasing the molecular weight of small proteins past the kidney elimination threshold [322]. Recently, promising results were obtained using bioconjugation of a water soluble polymeric carrier (poly(vinylpyrrolidone-co-dimethyl maleic anhydride)) to bioactive cytokines for use in targeted cancer therapeutics. In this case the relative potency of the drugs was increased [323].

## **2.7 Oral Insulin Delivery**

As stated earlier, the incidence and costs of diabetes treatment for insulin-dependent diabetes is staggering [13]. In addition to the social, behavioral and

economic justifications for oral insulin delivery, there are physiological reasons. As parenteral insulin administration targets peripheral tissues, rather than the liver, the normal dynamics of insulin release are not replicated [324]. This causes a delay in the onset of action as well as the need for higher insulin dosing. When this concept is combined with the many justifications previously stated, the reasons why we have seen insulin used as a model drug in almost every oral protein delivery system and methodology are apparent. In addition to being a model drug for varied drug delivery methodologies, there have been many approaches designed specifically for oral insulin delivery. Several of these approaches are described below.

Back in 1986, targeted colonic delivery in rats was attempted by coating insulin with polymers that were cross-linked with azoaromatic groups to form a protective shield [325]. There have also been numerous attempts to improve enteral insulin pharmacokinetics by novel formulation. Promising hypoglycemic results compared to controls were observed in rats when insulin was delivered via soft gelatin capsules coated with the polyacrylic polymer, Eudragit and containing a mixture of surfactants [326]. Another novel approach was to use lipoidal dispersion of insulin in fatty acids. A reduction of blood glucose from 105 mg/dl to 75 mg/dl in 30 minutes was observed in rabbits when a palmitic acid system containing 5 U insulin/50 mg dispersion was administered orally [327]. Similarly, water-in-oil-in-water (W/O/W) insulin emulsions containing lipoidal enhancers have been evaluated in animal models and it was determined that the biological efficacy of the emulsion was largely dependent on the lipid that constituted the oily phase [328]. Another formulation approach was to deliver insulin in a solid formulation consisting of



insulin mixed with cholate and soybean trypsin inhibitor. This approach demonstrated decreased blood glucose levels in dogs of greater than 20% of the initial value with the peak effect occurring after 60-140 minutes and lasting for more than 90 minutes [329]. Thiolated chitosan-insulin formulations of tablets and nanoparticles have also been studied in rats. In the case of the chitosan tablets given to non-diabetic rats, decreasing glucose levels were observed for 24 hours and in the case of chitosan-insulin nanoparticles, prolonged hypoglycemic effects of up to eleven hours were observed [287, 330].

### **2.7.1 Carrier systems for Insulin**

As we have seen there are many carrier systems developed for oral protein delivery and most have employed insulin as a model drug. In summary, carrier systems for oral insulin delivery have included polymeric delivery systems, such as hydrogels, micro- and nanoparticles, biodegradable polymers, liposomes and micelles, mucuadhesive polymers and bioconjugated polymer-protein formulations. Within each of these subsets, varying polymer chemistries have been applied. In addition, many of these systems have undergone optimization attempts via the incorporation of protease inhibitors or via efforts to increase mucoadhesion.

There are several additional cases of targeted insulin delivery systems that have not yet been discussed. Several insulin derivatives using polyethylene glycol (PEG) have been investigated. In the case of calcium phosphate-PEG-insulin-casein (CAPIC) particles, hypoglycemic effects of 50% of control were seen over the first 3 hours post dosing in diabetic mice [331]. Similarly, another PEGylated insulin approach utilized insulin-monomethoxypoly(ethylene glycol) derivatives prepared by

the preparation of mono- and di-terbutyl carbonate insulin derivatives demonstrated a 40% reduction in blood glucose within 3 hours of treatment [332].

Lastly, an additional novel approach is the use of an intestinal patch for enteral delivery. The concept is to concentrate insulin into a mucoadhesive patch that attaches to the intestinal mucosa allowing the patch to concentrate insulin locally for better absorption while still protecting the drug from enzymatic degradation. In one study, patches were prepared from Carbopol 934, pectin and carboxymethylcellulose at a weight ratio of 1:1:2. While a significant hypoglycemic effect was observed in rats *in situ*, the ability of the patches to remain attached to the intestinal wall decreased with increasing fluid volume in the intestines [333]. I feel that if adequate adhesion profiles are obtained with this type of approach and if one-directional insulin diffusion from the patches can be achieved, this approach could be very promising.

### **2.7.2 P(MAA-g-EG) Hydrogels**

The focus of this lab over the last two decades has been on a novel pH-sensitive complexation hydrogel system. As previously defined, hydrogels are water-swollen, crosslinked polymeric structures produced by the reaction of polymeric monomers or by association bonding [334]. The chemical and physical cross-links provide the network structure and physical integrity of the hydrogel while also rendering them insoluble. They are rubbery, can swell in the presence of some biological fluids and are highly biocompatible. These properties allow hydrogels to resemble living tissues more closely than any other class of synthetic biomaterials [335]. Although useful in many applications, such as contact lenses and dental

materials, it as a carrier system for protein drugs that is the focus of this current work. Specifically, a P(MAA-g-EG) hydrogel system will be detailed through the rest of this section.

### 2.7.2.1 Hydrogel Theory

Before the P(MAA-g-EG) system can be discussed, a brief overview of hydrogel parameters should be understood. The network permeability and swelling behaviors of a hydrogel network are important characteristics in the evaluation of a polymeric gel as a controlled release system. These aspects are mostly determined by the chemical nature of a polymer. As you may recall, an idealized hydrogel was presented in Figure 4 with  $M_c$  defined as the molecular weight of the polymer chains between cross-links and  $\zeta$  defined as the network mesh size, which is a measure of porosity literally defined as the distance between consecutive cross-links. The polymer volume fraction  $v_{2s}$  is another important parameter and is defined as the polymer volume fraction in the swollen state. Mathematically,  $v_{2s}$  can be defined as the ratio of the volume of the polymer ( $V_p$ ) to the volume of the swollen gel ( $V_{gel}$ ) as shown in equation 2.10.

$$v_{2s} = V_p / V_{gel} = 1/Q \quad (2.10)$$

Here  $Q$  is defined as the polymer fraction of polymer in the gel, which is a measure of how much fluid the hydrogel can incorporate into its structure when completely swollen. This quantity can be obtained experimentally from equilibrium swelling experiments. The degree of cross-linking in a hydrogel,  $X$ , is defined as the ratio of the molecular weight of the repeating units making up the polymer chains,  $M_o$ , to the molecular weights of the polymer chains between cross-links as shown below.

$$X = M_o / 2M_c \quad (2.11)$$

The ability of a hydrogel to swell and contain a fluid enables the hydrogels to take up, hold and release drugs. This essential property is enabled by ionic hydrogels. These hydrogels contain pendent groups that are cationic or anionic in nature. For anionic gels, such as the P(MAA-g-EG) system, the side groups are unionized below the  $pK_a$  and the swelling is governed by the thermodynamic compatibility of the polymer and swelling agent. Above the  $pK_a$  however, the pendent groups are ionized and the gels swell substantially due to the creation of a large osmotic swelling force created by higher concentration of ions present as shown in Figure 2.8. The opposite is true for cationic hydrogels. The relationship of the hydrodynamic radius,  $d_H$ , of a specific drug to the network pore size,  $\zeta$ , is an essential design parameter was presented earlier in Figure 2.4. Significant work has been done by Peppas et al to understand the detailed correlations between mesh size and the equilibrium degree of swelling of polymeric networks [336]. Lastly, the equations for diffusion of a drug from a macroporous hydrogel can be defined in terms of an effective diffusion coefficient,  $D_{eff}$  and the partition coefficient,  $K_p$ , as in Eqn 2.9 where  $D_{iw}$  is the diffusion coefficient of the solute in the pure solvent,  $\varepsilon$  is the network porosity and the network tortuosity is  $\tau$ .

$$D_{eff} = D_{iw} (K_p \varepsilon / \tau) \quad (2.12)$$

#### 2.7.2.2 Synthesis and Properties of P(MAA-g-EG) Hydrogels

Through the work of Lowman, Peppas et al the methods to synthesize P(MAA-g-EG) hydrogels has been optimized from the earliest published methods

[337]. For the sake of this dissertation, the following approach has been utilized. Microparticles of P(MAA-g-EG) are prepared via free-radical solution photopolymerization of methacrylic acid, MAA, and poly(ethylene glycol) methacrylate, PEGMA. The monomers are mixed in appropriate molar ratios to yield a 1:1 ratio of MAA:EG units in the resulting hydrogel. The monomers are then diluted to a desired (wt./wt.) of the total monomers with a 1:1 (vol./vol.) mixture of ethanol and water. Tetraethylene glycol dimethacrylate, TEGDMA, is then added as a cross-linking agent at  $X = 0.075$  moles TEGDMA per MAA. DMPA was added as the initiator in the amount of 1% weight of the monomers.

The resulting reaction mixtures are then formed into films by insertion into a glass mold that allowed for formation of a polymer film with a thickness of 0.8  $\mu\text{m}$ . The reactions are initiated and run to completion by exposing the monomer film to UV light (Ultracure 100, Efes, Buffalo, NY) at 1  $\text{mW}/\text{cm}^2$  at 365 nm for 30 minutes. The hydrogel films can then be removed from the molds and rinsed for 1 week in distilled  $\text{H}_2\text{O}$  (changed daily) to remove the un-reacted monomers and sol fraction [338]. The generic synthetic pathway is shown in Figure 2.9 below.

### **2.7.2.3 The *in vitro* and *in vivo* Characterization of P(MAA-g-EG) Hydrogels for Oral Insulin delivery**

The *in vitro* characterization of this novel hydrogel system can be broken down into two domains; the physical characterization of the polymer itself and the characterization of the biological activity of the polymer in *in vitro* and *in situ* biological models. A chronological progression of the progress of the *in vitro* characterization of this novel complexation hydrogel system follows.

As previously stated, the abilities of a hydrogel carrier system to take up, carry and release drug are key design criteria. In the case of the P(MAA-g-EG) system, extensive work has been done. The earliest efforts involved loading hydrogel particles with porcine insulin. The technique was to simply soak hydrogel particles in an insulin solution at pH 7.4 for 24 hours. It was determined that approximately 95% of the insulin in the solution was entrapped within the polymer. After drying and final processing, the particles were loaded into gel capsules and administered orally to male Wistar rats in which diabetes had been induced by injection of streptozotocin several weeks earlier. This study demonstrated an absolute oral bioavailability of ~4% in healthy rats that had been dosed orally at 50 IU/kg [339]. It was also determined that the hypoglycemic effects were dose related.

Later that same year, the mucoadhesive and drug uptake and release properties of the P(MAA-g-EG) system was studied. Using drugs of various sizes, it was determined that the diffusion of a protein drug through a swollen polymer was significantly hindered by the presence of cross-links as well as other physical obstructions in the polymer network. The mucoadhesive behavior of the gels was studied as a function of force and displacement for gels at different pH values. It was determined that the adhesive force was significantly higher at higher pH indicating that the free PEG chains may be serving as anchors to mucin under those conditions. Lastly the fractional release of insulin over time was documented for PEG chains of molecular weight 1000 in buffered saline solutions following dissolution in simulated gastric fluid for several hours demonstrated the expected gastric protection [337].

A similar pH-sensitive glycopolymer system developed by free-radical polymerization of methacrylic acid and 2-methacryloxyethyl glucoside, using tetra(ethylene glycol) dimethacrylate as a cross-linking agent was physically evaluated in detail. The swelling behavior was studied as a function of pH and copolymer composition. It was determined that the transition between the swollen and collapsed states occurred at pH 5 and that the mesh sizes of the hydrogels were 18-35 Å in the collapsed state (pH 2.2) and were between 70 and 111 Å at pH 7 in the swollen state [340]. It was also determined that the swelling ratio of the polymers decreased as the cross-linking ratio of the copolymers increased.

The physiochemical behavior and potential cytotoxicity of a nanosphere presentation of the P(MAA-g-EG) system was then evaluated in detail. Cytotoxicity was determined using an indirect measurement via a colorimetric assay and directly via a trypan blue exclusion method. The colorimetric assay demonstrated that Caco-2 cell monolayers remained more than 95% viable relative to controls. The cell counting method produced less precise and predictable variables due to cell separation and other complexities of that method. Transepithelial electrical resistance measurements (TEER) of the cell monolayers demonstrated that contact with the hydrogels did disrupt the cell monolayer. It was theorized that this was due to the opening of tight junctions by the chelation capabilities of these gels. The effect was found to be related to the amount of methacrylic acid in the hydrogels and was reversible [341]. The resulting conclusion of this study was that this complexation hydrogel system had the ability to enhance enteral insulin absorption via the paracellular route due to this ability to reversibly open tight junctions. Similar results

were obtained by Foss in 2004 using a P(MAA-g-PEG) hydrogel system [342]. In this paper, a correlation between decreasing TEER values and increasing insulin transport was determined to indicate paracellular transport. It was also shown that the hydrogels had a negligible effect on NADPH production by the cells, an indication of low or negligible toxicity. In addition, the relationship on monomer feed ratios was studied and it was determined that ratios of 1:1 and 2:1 were optimal when compared to monomer feed ratios of 3:1 and 4:1. That said, with respect to the mechanism of insulin transport, as the Caco-2 cells do have the insulin receptor, transcellular transport cannot be ruled out as a contributor.

In a similar Caco-2 study, microgels composed of cross-linked copolymers of Poly(acrylic acid) and Pluronics were evaluated as permeation enhancers for doxyrubicin. It was shown that the microgels inhibited P-gp-mediated doxyrubicin efflux and enhanced the passive influx. In this case it was determined that no meaningful enhancement of paracellular transport was observed [343].

In 2003, the next body of work on the P(MAA-g-EG) system involved study of the in vitro release behavior and the stability of insulin within the delivery system. A rapid release of insulin from the polymer was observed at pH 6.5 when the pH was titrated up from pH 2.2. It was also shown that the biological activity of insulin decreased as the molecular weight of the PEG component was increased. Lastly, PEG chains of molecular weight 200 demonstrated the best protective and release kinetics [344].

The influence of the reaction solvent content was studied in detail in late 2003. The effects of network morphology on the insulin delivery characteristics of



the system were also investigated. By preparing gels with greater reaction solvent contents, it was shown that the polymer mesh size was an increasing function of solvent content [338]. Similarly it was demonstrated that gels prepared with higher solvent content were capable of swelling to a greater degree. The pH-based protective capabilities were confirmed once again and insulin release rates were also increased with the solvent content of the reaction mixture. Closed loop rat studies showed that the insulin relative bioavailabilities were also a weak function of reaction solvent content, ranging from 4.6% for a polymer with a solvent fraction of 66.3% to 7.2% bioavailability for polymer with a solvent fraction of 22.3%.

The effects of the molecular weight of the PEG chains in the P(MAA-g-EG) hydrogel system and the effects of microparticle size on TEER and insulin transport in caco-2 cells have also been studied [345]. It was determined that TEER values were not a function of PEG molecular weights but that decreased microparticle sizes and shorter PEG chain lengths did lead to higher insulin permeability values in the Caco-2 model. This influence of sub-chain molecular weights was also studied by Ostroha et al [346]. In this study it was determined that the maximal degree of hydrogel swelling did increase weakly with sub-chain molecular weight. It was also determined that salt concentration has a significant effect on swelling at pH ranges near the transition value. This is an important event as it is the first documented detail on the effects of osmolarity on this delivery system. Lastly, it was shown that the incorporation of water-soluble uncharged pendant chains can increase the driving force for swelling without affecting the sub-chain elastic restoring force. Similar work on the effects that varying PEG molecular weights have on swelling and release

were presented by Peppas. In this work, the effects of PEG chain molecular weight on mucoadhesion was also studied using a tensile tester approach. It was determined again that the polymer is more mucoadhesive at high pH ranges due to the ability of free PEG chains to serve as anchors to mucin under these conditions [347].

The effects of particle size on the enteral absorption of insulin was detailed by Morishita et al [348]. Using an *in situ* closed loop procedure in rats, the hypothesis that smaller ILP particles would provide better insulin transport via improved mucoadhesion was confirmed with particles of not greater than 43  $\mu\text{m}$  in diameter showing the most pronounced hypoglycemic effects. It was also shown that the greatest effect was obtained when the particles were placed in the ileal segment when compared with analogous dosing in the jejunum. Lastly, it should be noted that much of the above work up to this time has been documented in an excellent review article by Peppas et al [349].

The most recent work on the P(MAA-g-EG) hydrogel system has focused on using animal models to determine the absolute and relative bioavailability of insulin delivered from the system. As previously discussed, most of the earlier work in animals have been *in situ* studies, which necessitate the introduction of many non-ideal variables such as deep anesthesia, significant handling stress, prolonged fasting, and bypass of the upper GI system just to name a few. Well-designed animal studies can remove or reduce the effects of most of these variables. In a recent publication, it was demonstrated that insulin-loaded polymer microparticles (ILP) with diameters < 53  $\mu\text{m}$  composed of a 1:1 molar ratio of MAA:EG units demonstrated a relative bioavailability of 9.5% when compared to subcutaneous insulin injection in healthy

rats. The associated pharmacodynamic study demonstrated the ability of the ILP system to suppress post-prandial glucose elevations in a multiple dose study [350]. While these results are promising, the fact that the animals were fasted extensively prior to the study raises some concerns. As previously discussed in an earlier section, the biochemical makeup of the intestinal brush border and intestinal motility vary significantly with changes in feeding times. It can be assumed that this study was conducted under fairly non-physiological conditions.

#### **2.7.2.4 The *in vitro* and *in vivo* Characterization of P(MAA-g-EG) Hydrogels for Oral delivery of other drugs and other routes of administration**

Early work with this complexation hydrogel system evaluated the potential for the delivery of protein therapeutics other than insulin and by routes other than oral. In a paper by Makamura et al, the nasal delivery of budesonide, a derivative of prednisolone used for the treatment of asthma and seasonal rhinitis, was evaluated using the P(MAA-g-EG) hydrogels system as the protein carrier [227]. This drug has been previously demonstrated to have a low oral bioavailability of ~11%. Using hydrogel microparticles to deliver the protein nasally in rabbits, a bioavailability of ~84% was reported. More recently, a nanoparticle formulation of this system was evaluated in several *in vitro* transport and tumor models for its possible efficacy in delivering chemotherapeutic agents [351]. It was demonstrated that the polymer nanoparticles enhanced bleomycin transport across Caco-2 cell monolayers and that drug released from the nanospheres successfully induced DNA damage in the DLD-1 tumor model.

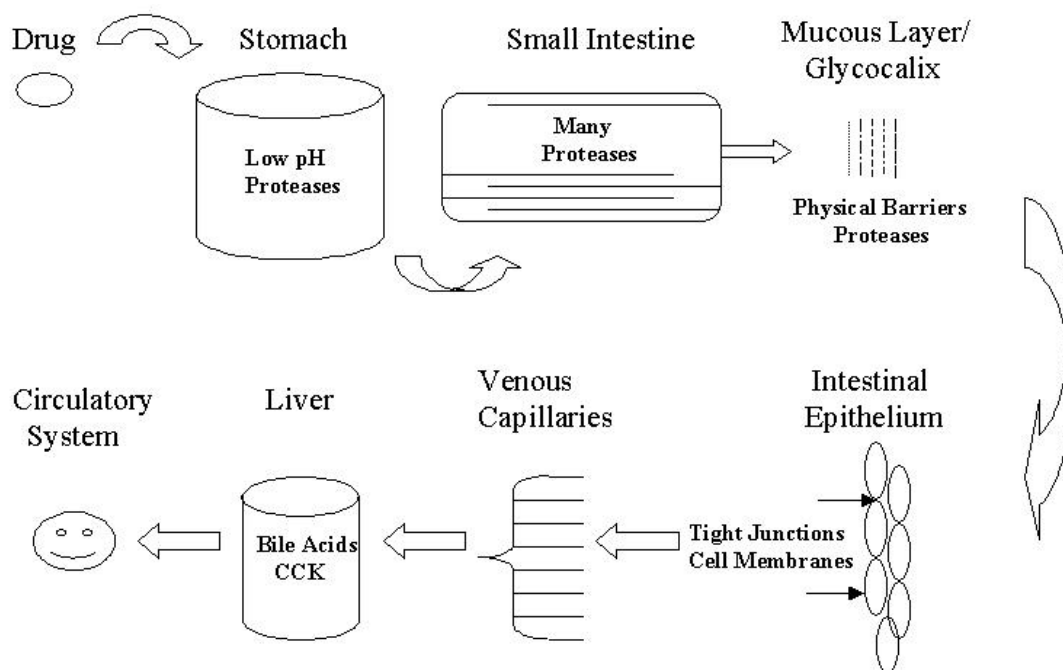
### 2.7.3 Oral Insulin Clinical Trial Results

As of August 2006, a search for oral insulin on the *clinicaltrials.gov* website returns 55 studies that are currently recruiting patients. For the most part, these studies are targeting Type II diabetic patients using various oral anti-diabetic agents. There are also several trials ongoing for the inhalant spray insulin formulations. It is startling, and somewhat disappointing, that, despite the hundreds of oral insulin approaches cited in this chapter, virtually none have made it to the clinic. There has been some successes however and this chapter will focus on the most recent, and promising, oral insulin clinical trial results.

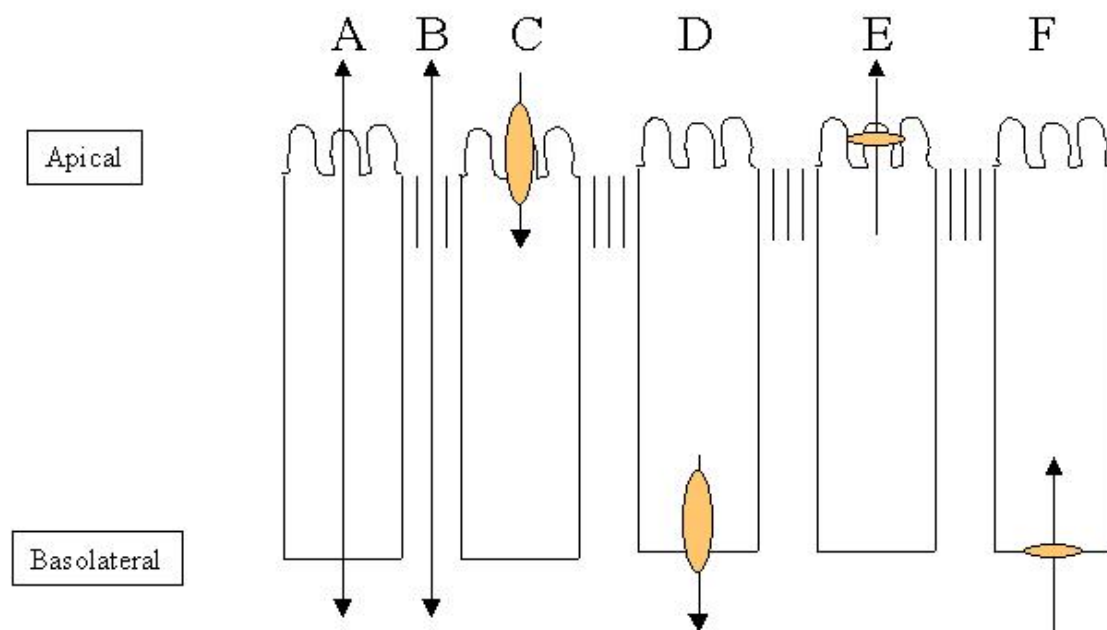
Hexyl-insulin monoconjugate 2 (HIM2) is an orally active insulin that is created by a site-specific oligomeric modification of recombinant human insulin [352]. An oligomer comprised of a PEG moiety plus an alkyl linker, is attached to B29 lysine. This formulation offers longer half-life, greater resistance to proteolytic damage and greater solubility in water and lipid-based media. In an open-label, non-randomized phase I/II clinical trial, 14 patients showed significant hypoglycemic effects from oral administration of this insulin formulation when added to a basal insulin regimen. In addition, no detectable adverse safety signals were observed. The utility of this formulation will most likely be tested next to evaluate the blood glucose control efficacy in the absence of basal subcutaneous insulin.

Another recent study compared the PK and PD of a single-dose oral insulin spray with subcutaneous insulin. In this small study using six healthy male volunteers, the oral spray demonstrated a higher  $C_{MAX}$  and shorter  $T_{MAX}$  than subcutaneous insulin, as well as faster time to peak glucose uptake [353]. These

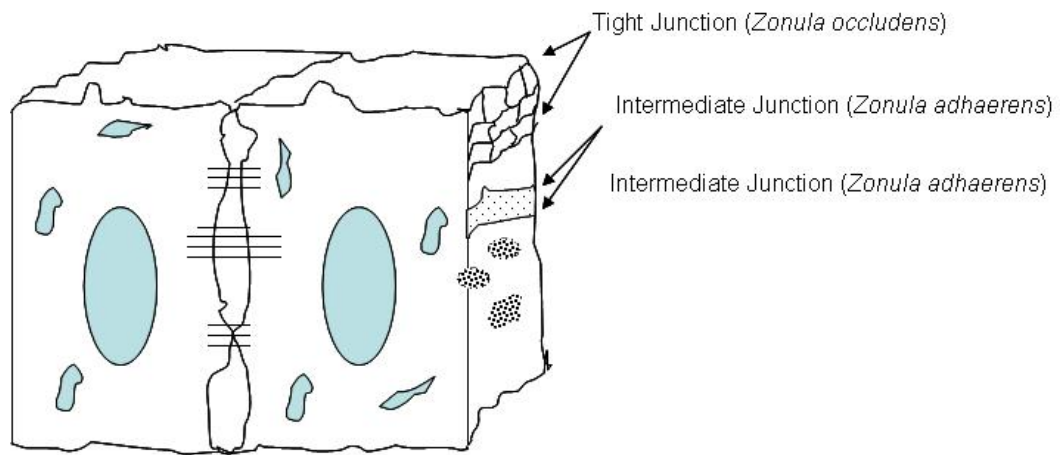
PK/PD results imply that this formulation may be helpful in managing meal-related blood sugar excursions. It must also be noted that four of the six subjects complained of a strange sensation and taste in their mouths and 3 subjects experienced mild dizziness. That said, all of the effects were of short duration and the vital signs of all patients remained stable throughout this study.



**Figure 2.1: The Oral Delivery Gauntlet**

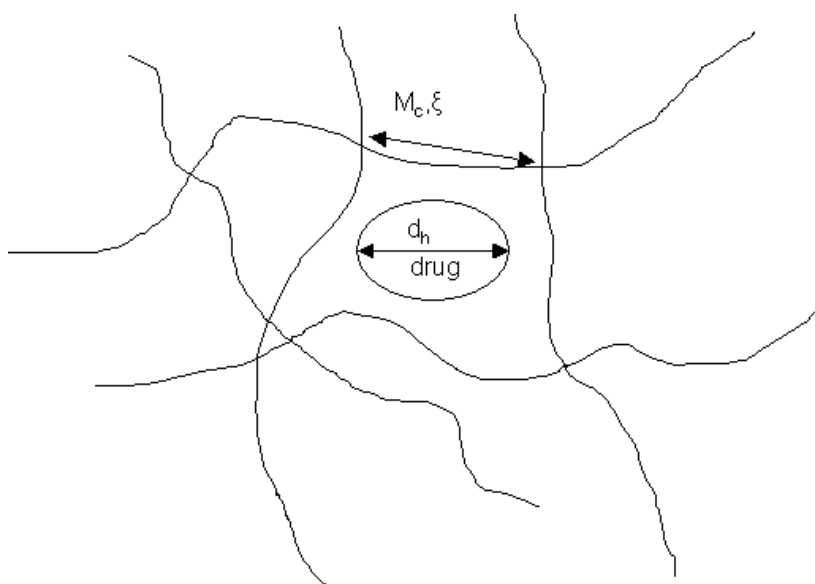


**Figure 2.2: Routes of Drug Transport Across Cellular Barriers (A) Passive Transcellular; (B) Passive Paracellular; (C and F) Carrier-mediated Uptake; (D and E) Carrier Mediated Efflux**

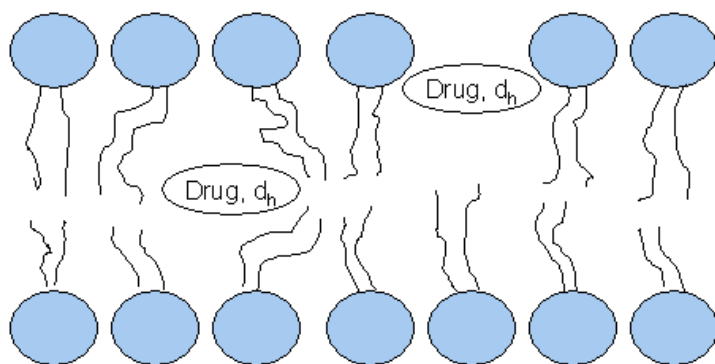


**Figure 2.3: Tight Junction Structure**

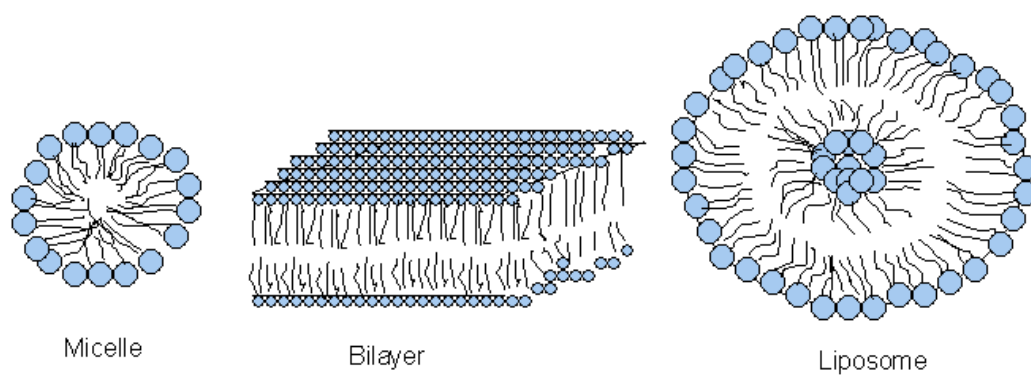




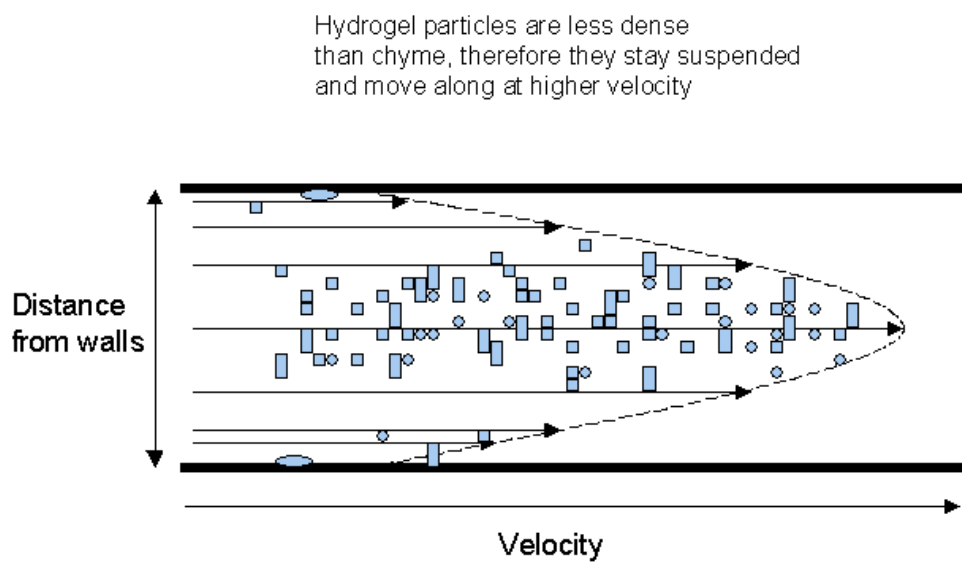
**Figure 2.4: Key Hydrogel Parameters**  $M_c$  is the molecular weight between crosslinks,  $\xi$  is the mesh size in Å,  $d_h$  is the hydrodynamic radius of a drug



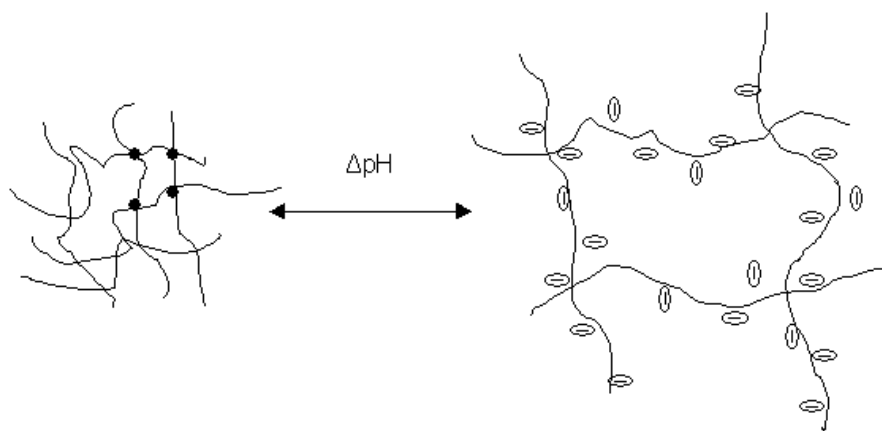
**Figure 2.5: Lipid Formulations**



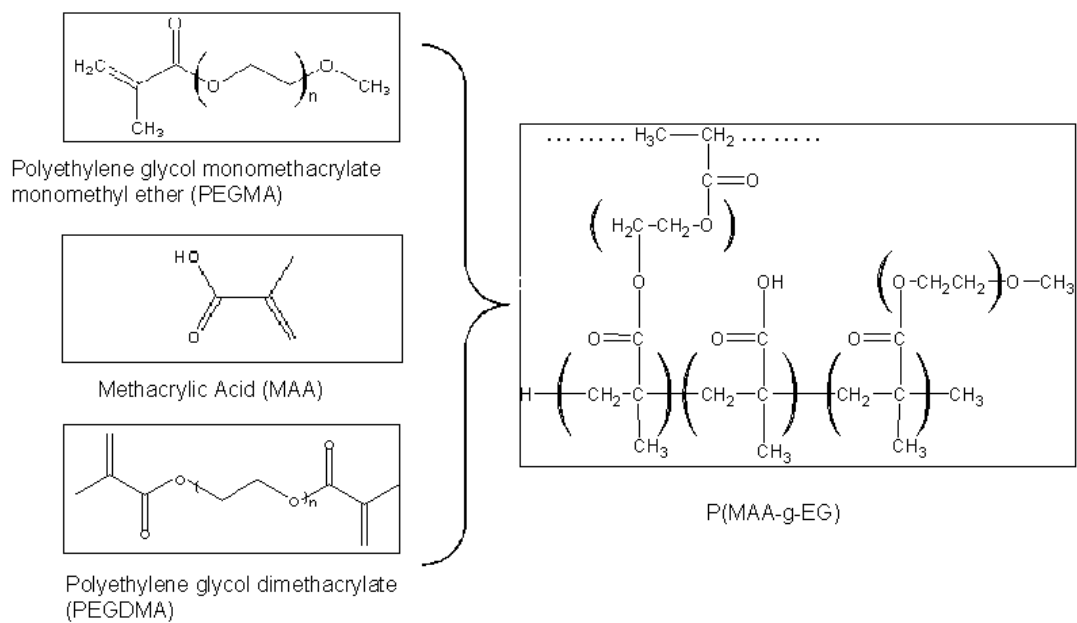
**Figure 2.6: Amphipathic Lipid Aggregates that Naturally Form in Water**



**Figure 2.7: Schematic of Flow through the GI tract**



**Figure 2.8: Swelling of an Anionic Hydrogel**



**Figure 2.9: P(MAA-g-EG) Hydrogel Synthesis**

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### CHAPTER 3: RESEARCH GOALS

The ultimate goal of this work is to support the development of an oral drug delivery system for protein therapeutics based upon complexation hydrogel technology by gaining a detailed understanding of its mechanisms of action. A novel pH-sensitive poly [methacrylic acid-grafted-poly (ethylene glycol)] hydrogel system has previously demonstrated enhanced insulin bioavailability in an *in vivo* model. The strategy of the current work included characterization of the *in vitro* transport enhancing effects of this system followed by a solid pre-clinical evaluation of the system. The approach can be parsed into 4 specific aims.

**Aim #1:** Select, develop and establish an *in vitro* model of gastrointestinal transport. Various models of gastrointestinal transport were studied. The Caco-2 cell line was selected as the optimal model of gastrointestinal transport. The cells were purchased and a viable culture line was established.

**Aim #2:** Establish transepithelial resistance measurement capability as a model of monitoring confluence of cellular cultures and tight junction integrity. Models of cell monitoring were studied and transepithelial electrical resistance monitoring (TEER) was selected and implemented. TEER monitoring was used to evaluate the performance of the cell cultures as well as to determine confluency.

**Aim #3:** Confirm the ability of hydrogel to protect insulin in the upper GI system, the ability to release insulin in the lower GI system via dissolution studies and

quantify the insulin transport capability of the hydrogel system via permeability studies. The ability of hydrogel to protect insulin in the upper GI system and the ability to release insulin in the lower GI system was demonstrated via dissolution studies in the USP models simulated gastric fluids containing active enzymes. The insulin transport capability of the hydrogel system was quantified via permeability studies

**Aim #4:** Develop a detailed pre-clinical profile of hydrogel system including proof of concept, dosing, toxicity and pharmacokinetic and pharmacodynamic profiling via an *in vivo* study. An *in vivo* pre-clinical evaluation of the hydrogel system in two species was utilized to determine the absolute bioavailability of insulin delivered orally via this system. These studies utilized an advanced intravenous-to-oral crossover design to enable the most accurate model of oral dosing obtained to date on this hydrogel system. Lastly, the pharmacokinetic and pharmacodynamic properties of insulin delivered via the P(MAA-g-EG) hydrogel system were detailed via pharmacokinetic modeling.

## CHAPTER 4: SYNTHESIS AND CHARACTERIZATION OF P(MAA-G-EG) HYDROGELS

### 4.1 Introduction

As previously discussed, hydrogels have been a focus of study in drug delivery and biomaterials applications for several decades. The particular focus in this lab has been the development of a novel pH-sensitive complexation hydrogel system. Significant strides have been made in hydrogel theory, practical synthesis and the physical and *in vitro* characterization of hydrogels.

Hydrogels were defined in the early 1940s as water-swollen, cross-linked polymeric structures produced by the reaction of one or more monomers or by association bonding such as hydrogen bonding and/or van der Waals interactions of the polymer chains<sup>1,2</sup>. Hydrogels can be classified in many ways by charge (neutral, anionic, cationic and ampholytic), by mechanical and structural properties (affine, phantom), by method of preparation (homopolymer networks, copolymer networks, multipolymer networks or interpenetrating polymer networks) or, lastly, by physical structural features (amorphous, semicrystalline, hydrogen-bonded, supermolecular network structures or hydrocolloidal aggregates)<sup>1</sup>. The key parameters that distinguish hydrogels physically and behaviorally have become much more understood.

Of particular importance is the relationship between the hydrogel mesh size ( $\xi$ ) and the degree of swelling within a polymer network. These parameters directly

influence the transport mechanisms of drugs and other molecules through the polymer networks. For example, it is known that the diffusion coefficient of a drug through a hydrogel decreases as the crosslinking density increases<sup>3</sup>. Similarly, it is known that the polymer molecular weight directly affects the drug release behaviors from hydrogel networks<sup>4</sup>. Quantitatively, hydrogel structure and behavior can be characterized by several key relationships. The polymer volume fraction ( $v_{2s}$ ) is the ratio of the polymer volume ( $V_p$ ) to the volume of the swollen gel ( $V_{gel}$ ) and is defined by equation 2.10.

$$v_{2s} = V_p / V_{gel} = 1/Q \quad (2.10)$$

where  $Q$  is defined as the polymer fraction of polymer in the gel, which is a measure of how much fluid the hydrogel can incorporate when swollen to its maximum state.

This parameter can be obtained experimentally from equilibrium swelling experiments. The degree of crosslinking in a gel ( $X$ ) is defined by equation 2.11

$$X = M_o / M_c \quad (2.11)$$

Where  $M_c$  is the molecular weight of the polymer chains between crosslinks and  $M_o$  is the molecular weights of the polymer units that make up the polymer chains<sup>5</sup>. The network pore size can be obtained from equation 4.1 where  $r_0^2$  is defined as the unperturbed end-to-end distance of the polymer chains between cross-linking points

$$\zeta = \alpha(r_0^2)^{1/2} \quad (4.1)$$

and the elongation ( $\alpha$ ) can be related to the swollen polymer volume fraction, assuming isotropic swelling.



The swelling and release behaviors of hydrogels are also critical parameters.

In the case of ionic hydrogels, swelling behavior can be described in terms of the free energy ( $\Delta G$ ) of the system as shown in equation 4.2.

$$\Delta G = \Delta G_{mix} + \Delta G_{gel} + \Delta G_{elastic} + \Delta G_{ion} \quad (4.2)$$

Which presents the system free energy as a summation of free energy of mixing, elasticity and ionic potential. In macroporous hydrogels, gels with pore sizes between 0.1  $\mu\text{m}$  and 1  $\mu\text{m}$ , diffusion can be characterized in terms of the diffusion coefficient of the solute in pure solvent, as well as the network porosity and tortuosity as shown in equation 2.12.

$$D_{eff} = D_{iw} (K_p \varepsilon / \tau) \quad (2.12)$$

Lastly, many of these parameters can be obtained experimentally, either by direct measurement or by application of some of the above relationships. Specifically, the diffusion coefficient can be obtained via membrane permeation, absorption/desorption studies, scanning electron microscopy (SEM), infrared spectroscopy (IR) or quasi-elastic light scattering (QELS) and methods information of each of these approaches is readily available <sup>6</sup>.

In the current work, hydrogels were synthesized by free-radical solution photo-polymerization. In photo-initiated reactions, polymerization occurs when radicals are produced by UV and/or visible light. This can happen when a compound in the reaction undergoes excitation via energy absorption and decomposes into radicals or when the excited species interacts with a second compound to form radicals of one species via energy transfer or redox <sup>7</sup>. Crosslinking to form the hydrogel network occurs when the branches from the growing polymer chains

interact with one another. The polymer growth is eventually terminated by the destruction of the reactive center by an appropriate reaction.

In order to understand the potential for a hydrogel system to act as a protein drug carrier, detailed characterization is required. As previously mentioned, the volume swelling ( $Q$ ) and the weight swelling ratio ( $q$ ) can be obtained experimentally. Similarly, the hydrogel network structure and the loading and release kinetics of a given hydrogel system can be obtained using *in vitro* techniques<sup>8</sup>.

In the current work, P(MAA-g\_EG) hydrogels were synthesized and physically characterized in preparation for detailed *in vitro* and *in vivo* pharmacokinetic and pharmacodynamic study.

## 4.2 Experimental Section

### 4.2.1 Materials

Methacrylic acid (MAA), pepsin, pancreatin and insulin (bovine and human) were purchased from Sigma Aldrich (St. Louis, MO). Dimethoxy propyl acetophenone (DMPA) was purchased from Aldrich (Milwaukee, WI). The MAA was purified via packed column of DE-HIBIT 200 (Polysciences, Warrington, PA) in order to remove the inhibitor (hydroquinone monomethyl ether). Methoxy-terminated poly(ethylene glycol) monomethacrylate 1000 (PEGMA), and tetraethylene glycol dimethacrylate (TEGDMA) were all purchased from Polysciences (Warrington, PA). All chemicals were used as supplied unless noted as exceptions below.

#### 4.2.2 Preparation of P(MAA-g-EG) Hydrogels

Microparticles of P(MAA-g-EG) were prepared via free-radical solution photo-polymerization of MAA and PEGMA<sup>8</sup>. The monomers were mixed in appropriate molar ratios to yield a 1:1 ratio of MAA:EG units in the resulting hydrogel. The monomers were then diluted to 44.7% (wt./wt.) of the total monomers with a 1:1 (vol./vol.) mixture of ethanol and water. TEGDMA was added as a cross-linking agent at  $X = 0.075$  moles TEGDMA per MAA. DMPA was added as the initiator in the amount of 1% weight of the monomers.

The reaction mixtures were pipetted into a glass mold that allowed for formation of a polymer film with a thickness of 0.8  $\mu\text{m}$ . The reactions were initiated and run to completion by exposing the monomer film to UV light (Ultracure 100, Efes, Buffalo, NY) at 1  $\text{mW}/\text{cm}^2$  at 365 nm for 30 minutes. The hydrogel films were removed from the molds, rinsed for 1 week in distilled  $\text{H}_2\text{O}$  (changed daily) to remove the un-reacted monomers and sol fraction.

#### 4.2.3 Insulin Loading

Hydrogel films were dried under vacuum at 37°C and then pulverized into fine particles using a mill (Bell Art Products, Pequannock, NJ). The particles were further ground using a mortar and pestle and passed through sieves with a particle size cutoff of 43  $\mu\text{m}$ . An insulin solution was prepared by dissolving 10 mg of bovine insulin in 20 ml of PBS (pH = 7.4). The crushed polymer microparticles were then dispersed in

the insulin solution at 37°C and stirred at 300 rpm. After 6 hours, a 0.2ml aliquot was removed for loading analysis via syringes equipped with 0.45 µm membrane filters and 10 ml of 0.1N HCl was added to the solutions to collapse the polymers, effectively entrapping insulin. The resulting solutions were filtered with cellulose acetate/cellulose nitrate filters (0.45 µm, Fisher Scientific, PA). The polymers were further washed with 100 ml 0.1N HCl and 100 ml of de-ionized water. All glassware was siliconized via treatment with Sigmacote® (Sigma). The resulting insulin-loaded polymer (ILP) was dried in a lyophilizer (Virts, Gardiner, NY) at -80°C and stored at -20°C. HPLC was used to determine the insulin content of the wash solutions and the resulting insulin content of the ILP was determined via a simple mass balance.

As a negative control, Dummy-loaded polymer (DLP) was also prepared. These samples were brought through the exact same process as the ILP above except the loading solution contained no insulin. This preparation produced un-loaded polymer samples that had been through the same treatments as ILP, incubation and network collapse with 0.1N HCl etc., resulting in a control sample that has the same surface composition as ILP.

#### **4.2.4 HPLC**

A Waters Symmetry® 300 column was used with a gradient of 25:75 acetonitrile/water to 45:55, including 0.1% TFA, over 20 minutes employed as the mobile phase. Samples were injected into a Waters 2690 separations module equipped with a 996 Photodiode Array detector at a 1ml/min flow rate

#### 4.2.5 Insulin Release

To quantify the ability of ILP to release insulin, 10 mg ILP samples were dispersed via stirring at 300 rpm in 20 ml PBS in a siliconized vessel at 37°C. Aliquots of 0.2 ml were taken via filter-equipped syringe at set time intervals. The volume was kept constant via immediate replenishment with an equal volume of warm PBS. The insulin concentrations were determined via HPLC.

#### 4.3 Results and Discussion

There were essentially three hydrogel synthesis and characterization campaigns in this dissertation. The first campaign produced the hydrogel particles for the simulated gastric fluids and Caco-2 cellular transport studies. Table 4.1 shows the results of these efforts. The remaining 2 synthesis campaigns were for the rodent and canine *in vivo* studies respectively and the results of those efforts are presented in Tables 4.2 and 4.3 respectively.

As shown in these tables, the average loading percent was approximately 6% for all three campaigns and all synthesis were conducted in small batches. To ensure equivalent dosing, an average loading percent was assumed and samples were pooled.

#### 4.4 Conclusions

The primary goal of this work was to advance the current methods and develop new methods for the *in vitro* and *in vivo* characterization of the P(MAA-g-

EG) hydrogel system. To this ends, optimal hydrogel preparation methods were chosen based on the existing body of knowledge and were not varied throughout this research project in order to ensure consistency. The result is a body of scientific work that can be applied to newer hydrogel formulations as they are developed.

**Table 4.1: ILP Preparations for the Simulated Gastric Fluid Digestions and the Caco- 2 Permeability Studies**

<b>Batch</b>	<b>Amount</b>	<b>Formulation Loading</b>	<b>Insulin</b>	<b>Insulin</b>
	(g)	(%)	(g)	(units)
A	3.9	5.76	0.23	8390
B	4.1	4.83	0.19	6932
C	3.9	5.98	0.23	8390
D	4.0	6.06	0.24	8756
F	4.1	6.22	0.26	9485
		Total:	1.16	41953

**Table 4.2: Rodent *in vivo* Study ILP Synthesis Summary**

Insulin Loaded and Dummy Loaded Polymer Sample Summary		
	Amount (mg)	Formulation Loading (%) <sup>1</sup>
Dummy Loaded Polymer (DLP)	667.7	0.00
Insulin Loaded Polymer Batch Identifier (ILP):	Amount (mg)	Formulation Loading (%) <sup>1</sup>
ILP A1	111.2	4.72
ILP A2	110.7	4.77
ILP B1	113.1	5.66
ILP B2	111.2	5.49
ILP C1	111.1	5.63
ILP C2	114.1	5.64
ILP D1	$\approx 110^2$	5.29
ILP D2	$\approx 110^2$	5.38
ILP E1	115.8	5.42
ILP E2	118.6	5.45
ILP F1	115.3	5.44
ILP F2	114.2	5.64
ILP G1	110.6	5.73
ILP G2	117.9	5.72
ILP H1	120.0	5.89
ILP H2	125.6	5.56
	TOTAL: 1829.4 mg	AVG: 5.46 %

1 – Weight % of insulin in ILP

2 – Final weight of ILP approximated



**Table 4.3: Canine *in vivo* Study ILP Synthesis Summary**

<b>Batch</b>	<b>Amount</b>	<b>Loading Efficiency</b>	<b>Formulation Loading</b>	<b>Insulin</b>	<b>Insulin</b>
	(g)	(%)	(%)	(g)	(units)
E	3.76	85.6	5.76	0.22	8026
F	3.95	71.7	4.83	0.20	7067
G	3.93	89.0	5.98	0.23	8700
H	3.93	90.5	6.06	0.24	8824
I	3.91	92.9	6.22	0.24	8990
			Total:	1.12	41607

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## **CHAPTER 5: COMPLEXATION HYDROGELS FOR ORAL PROTEIN DELIVERY: AN *in vitro* ASSESSMENT OF THE INSULIN TRANSPORT ENHANCING EFFECTS FOLLOWING DISSOLUTION IN SIMULATED DIGESTIVE FLUIDS**

### **5.1 Introduction**

The interest, demand and market potential for advanced drug delivery systems for hormones, antibodies and other protein drugs has been well documented <sup>1</sup>. Of particular interest is the ability to deliver proteins orally. Ideally, oral protein delivery has the potential to increase patient compliance, lower medical treatment costs and provide superior pharmacokinetic profiles <sup>2</sup>. The primary barrier to effective oral protein delivery is the ability to obtain adequate bioavailability due to drug degradation by proteolytic enzymes and poor transport across epithelial membranes as it passes through the gastrointestinal tract.

One promising approach to oral protein delivery is the use of hydrogel technology, which has been studied extensively over the last 2 decades <sup>3-9</sup>. Of particular interest are pH-sensitive complexation hydrogels systems, which have demonstrated significant promise in oral delivery, especially in the specific application of oral insulin delivery <sup>10-13</sup>. The poly(methacrylic acid-g-ethylene glycol) (P(MAA-g-EG)) system developed through this work has demonstrated the ability to enhance insulin bioavailability in an *in situ* closed loop model compared to insulin alone <sup>12</sup>, however the specific mechanism of transport enhancement is unknown.

*In vitro* cell culture models are commonly used in the study of drug absorption mechanisms<sup>14</sup>. The Caco-2 model, a human intestinal epithelial cell line, is thought to satisfy the need for an oral absorption model that predicts *in vivo* situations in a fast and efficient manner<sup>15</sup>. In these models, pure cell lines are grown to confluency on a semi-permeable membrane. Drug or drug formulations are then placed on the apical side of the monolayer and transport across the monolayer is determined via measurement of drug substance on the basolateral side of culture vessel. The Caco-2 model has been used to study the transport of many large molecule drug substances including insulin<sup>16-18</sup>. Further, the interest and utility of this model have led to significant optimization and study<sup>19-23</sup>. The application of these optimized models facilitates a detailed understanding of intestinal drug transport.

Intestinal absorption of a compound can occur via transcellular passive permeability, carrier-mediated transport or paracellular passive permeability<sup>24</sup> or by the other routes shown in Figure 2<sup>25</sup>. In general, there are very few hydrophilic compounds that can cross the epithelial barrier via the transcellular route, so the paracellular or junctional pathway is the only alternative pathway that is available to provide adequate drug for systemic bioavailability.

The specific mechanisms of absorption are of interest in the study of this complexation P(MAA-g-EG) hydrogel system as an *in vivo* model has demonstrated higher bioavailability in treatment with insulin-loaded-polymer samples than in the case of insulin dosing alone<sup>12</sup>. In these studies, *in situ* closed loop sections of rat intestine were dosed with insulin alone and insulin-loaded polymer (ILP). The ILP samples produced bioavailability of 4.6 – 7.4 % while the bioavailability obtained

from insulin alone was significantly less than 1%. These impressive results drive the need to understand exactly how the ILP is enhancing transport. Transport studies using Caco-2 cells and a similar hydrogel system, have quantified some baseline insulin permeability values and have generated the hypothesis that the hydrogel system may be enhancing insulin transport via the paracellular route <sup>18</sup>. The hypothesis surmises that the hydrogels can bind calcium in the cell media and, as tight junction integrity is calcium dependent; tight junctions ‘loosen’ allowing the drug to pass through. Experiments designed to measure and monitor tight junction integrity using transepithelial electrical resistance measurements (TEER) across media of varying calcium concentrations support the  $\text{Ca}^{2+}$  dependency of tight junction behavior <sup>26,27</sup>. That said, it has long been known that the Caco-2 cell line contains the insulin receptor, and, although the specific purpose of the receptor is unknown, a possible role of active transport in the insulin transport enhancing effects of this hydrogel system cannot be completely dismissed<sup>28</sup>.

Lastly, these studies confirmed that the hydrogel systems were not cytotoxic to Caco-2 cells, which rules out cellular toxicity damage as a potential mechanism of action to explain the bioavailability data but does not rule out irreversible changes to tight junction macro and protein structure, which could be interpreted as toxicity-based mechanisms. In fact,  $\text{Ca}^{2+}$  depletion has been shown to induce significant and global changes in cells, which include the disruption of actin filaments and adherent junctions as well as diminished cell adhesion some of which have been reported to lead to unacceptable side effects <sup>29,30</sup>. As a result, the possibility of a role of active

transport and the optimization of receptor mediated epithelial membrane transport should not only be seen as possible but also as desirable.

In order to further understand and quantify the potential of the P(MAA-g-EG) to improve the oral bioavailability of insulin, two questions must be answered more thoroughly. First, a model that demonstrates the ability of this system to protect insulin from proteolytic damage in the upper gastrointestinal system is required. Initial work on developing this model is underway and recent work has demonstrated that this hydrogel system can protect insulin from proteolytic damage in simulated gastrointestinal fluids derived from extracts of stomach and intestinal contents from Wistar rats<sup>31</sup>. Second, the Caco-2 transport properties must be confirmed by samples that have passed through these models of gastrointestinal protection and extended to include other P(MAA-g-EG) formulations in the hope of confirming the proposed mechanisms of cellular transport.

As a model of gastrointestinal protection and *in vivo* release, P(MAA-g-EG) microparticles were exposed to a sequential series of dissolution studies utilizing simulated gastric fluid USP with pepsin (SGF) and simulated intestinal fluid USP with pancreatin (SIF). By the sequential exposure of hydrogel microparticles to SGF and SIF prior to the application of these microparticles to the Caco-2 transport model, aspects of the prior system characterization are brought together holistically to yield the most realistic *in vitro* model of an oral dose presented to date.

## 5.2 Experimental Section

### 5.2.1 Materials

Methacrylic acid (MAA), pepsin, pancreatin and insulin (bovine and human) were purchased from Sigma Aldrich (St. Louis, MO). Dimethoxy propyl acetophenone (DMPA) was purchased from Aldrich (Milwaukee, WI). The MAA was purified via packed column of DE-HIBIT 200 (Polysciences, Warrington, PA) in order to remove the inhibitor (hydroquinone monomethyl ether). Methoxy-terminated poly(ethylene glycol) monomethacrylate 1000 (PEGMA), and tetraethylene glycol dimethacrylate (TEGDMA) were all purchased from Polysciences (Warrington, PA).

### 5.2.2 Hydrogel Synthesis

Microparticles of P(MAA-g-EG) were prepared via free-radical solution photo-polymerization of MAA and PEGMA<sup>12</sup>. The monomers were mixed in appropriate molar ratios to yield a 1:1 ratio of MAA:EG units in the resulting hydrogel. The monomers were then diluted to 44.7% (wt./wt.) of the total monomers with a 1:1 (vol./vol.) mixture of ethanol and water. TEGDMA was added as a cross-linking agent at  $X = 0.075$  moles TEGDMA per MAA. DMPA was added as the initiator in the amount of 1% weight of the monomers.

The reaction mixtures were pipetted into a glass mold that allowed for formation of a polymer film with a thickness of 0.8  $\mu\text{m}$ . The reactions were initiated and run to completion by exposing the monomer film to UV light (Ultracure 100, Efos, Buffalo, NY) at 1 mW/cm<sup>2</sup> at 365 nm for 30 minutes. The hydrogel films were

removed from the molds, rinsed for 1 week in distilled H<sub>2</sub>O (changed daily) to remove the un-reacted monomers and sol fraction.

### 5.2.3 Insulin loading

Hydrogel films were dried under vacuum at 37°C and then pulverized into fine particles using a mill (Bell Art Products, Pequannock, NJ). The particles were further ground using a mortar and pestle and passed through sieves with a particle size cutoff of 43 µm. An insulin solution was prepared by dissolving 10 mg of bovine insulin in 20 ml of PBS (pH = 7.4). The crushed polymer microparticles were then dispersed in the insulin solution at 37°C and stirred at 300 rpm. After 6 hours, a 0.2ml aliquot was removed for loading analysis via syringes equipped with 0.45 µm membrane filters and 10 ml of 0.1N HCl was added to the solutions to collapse the polymers, effectively entrapping insulin. The resulting solutions were filtered with cellulose acetate/cellulose nitrate filters (0.45 µm, Fisher Scientific, PA). The polymers were further washed with 100 ml 0.1N HCl and 100 ml of de-ionized water. All glassware was siliconized via treatment with Sigmacote® (Sigma). The resulting insulin-loaded polymer (ILP) was dried in a lyophilizer (Virts, Gardiner, NY) at -80°C and stored at -20°C. HPLC was used to determine the insulin content of the wash solutions and the resulting insulin content of the ILP was determined via a simple mass balance.

### 5.2.4 HPLC

A Waters Symmetry® 300 column was used with a gradient of 25:75 acetonitrile/water to 45:55, including 0.1% TFA, over 20 minutes employed as the



mobile phase. Samples were injected into a Waters 2690 separations module equipped with a 996 Photodiode Array detector at a 1 ml/min flow rate.

#### **5.2.5 Insulin Release**

To quantify the ability of ILP to release insulin, 10 mg ILP samples were dispersed via stirring at 300 rpm in 20 ml PBS in a siliconized vessel at 37°C. Aliquots of 0.2 ml were taken via filter-equipped syringe at set time intervals. The volume was kept constant via immediate replenishment with equal volume of warm PBS. The insulin concentrations were determined via HPLC.

#### **5.2.6 Digestion Studies in Simulated GI Media**

For these studies, simulated intestinal fluid (SIF) and simulated gastric fluid (SGF) with enzymes were prepared in accordance with USP standards. Briefly, SGF was prepared by dissolving 2.0 g sodium chloride and 3.2 g purified pepsin derived from porcine stomach mucosa (Sigma Aldrich), with an activity of 800-2500 units per mg of protein, in 7.0 ml of hydrochloric acid (37%) and sufficient DI water to make 1 L of solution. The pH was adjusted to 1.2.

To prepare the SIF, 6.8 g of monobasic potassium phosphate (Sigma Aldrich) was mixed and dissolved into water. Sodium hydroxide (Sigma Aldrich, 0.2N) and water were added, followed by 10.0 g pancreatin. The resulting solution was then brought to pH 6.8 with 0.2 N NaOH and diluted to a volume of one liter.

The SGF digestion was performed by warming 1 liter of SGF to equilibrium at 37°C and adding ILP while stirring at 150 rpm. The ratio of ILP to solution was

selected to simulate “sink” conditions. At time equal to 0, 20, 40, 60 and 120 minutes, a 0.2 ml aliquot was removed from the dissolution cell via a syringe equipped with a 0.45  $\mu\text{m}$  filter. Fresh SGF (0.2 ml) were back-flushed through the filter as media replacement and to return any hydrogel particles adhering to the filter. After 2 hours, the entire solution was centrifuged and the supernatant was removed. The particles were then washed serially via re-suspension in SGF, followed by centrifugation and supernatant removal two times. After the final collection, half the particles were rinsed in pH = 1.2 solution followed by rinsing in HBSS and quickly frozen to prevent polymer swelling. The particles were then lyophilized and stored at  $-20^{\circ}\text{C}$ .

The remaining particles were then subjected to SIF digestion. These particles were placed in 500 ml of SIF and stirred for 6 hours at 150 rpm and  $37^{\circ}\text{C}$ . Small aliquots (0.2 ml) were removed at 0, 30, 45, 60, 120, 240 and 360 minutes. SIF was back-flushed through the filters to maintain constant volume in the dissolution vessels and to return particles back into the solutions. After 6 hours, the particles were filtered, washed in SIF, centrifuged and recovered as per the SGF digestion. The samples were then washed in PBS (pH=6.8) followed by HBSS, lyophilized and stored at  $-20^{\circ}\text{C}$ .

### **5.2.7 Caco-2 Cell Monolayer Transport Studies**

Caco-2 cells were obtained from American Type Culture Collection (Rockville, MD). Caco-2 cells were grown in 75  $\text{cm}^2$  Corning flasks in Dulbecco's Modified Eagle Medium (1X, cat. #12430) which was enhanced with 10% fetal

bovine serum (v/v), 1% non-essential amino acids and 1% Pen Strep in an incubator at 37°C and controlled atmosphere of 90% relative humidity and 5% CO<sub>2</sub>. Cells were sub-cultured via trypsinization once to twice per week at a cut ratio of 1:3 or 1:4 and the media was changed at least once between passages. The cells were observed daily via microscope to ensure adequate growth and monitor for contamination.

Transport studies were performed in Corning, Transwell plates (Corning Life Sciences, Acton, MA. catalog #3491), in which the cells had been grown to confluency, typically 20-23 days and a TEER value of greater than 350  $\Omega$  cm<sup>2</sup>. Cell monolayers were exposed to insulin alone (I), insulin in the presence of polymer (PI), polymer, which had been previously ‘loaded’ with insulin (ILP), and polymer alone as a negative control for insulin. In addition, ILP samples from the digestion studies were also applied. All cells used for these samples came from passages between 88 and 98.

Transepithelial electrical resistance measurements (TEER) were used as a measure of tight junction integrity and were performed using the EVOM Epithelial Volt-ohmmeter and STX2 Electrode systems from World Precision Instruments. The instrument was used as per manufacturer’s instructions and the electrodes were sterilized prior to use and rinsed frequently with diluted ethanol and cell medium to prevent cross-contamination.

In the TEER studies involving the P, I, PI and ILP samples, the growth media was removed from the cells which were then rinsed with Hank’s Balanced Salt Solution (HBSS, Invitrogen cat# 24020117). An initial TEER reading was obtained after a 30-60 minute incubation in HBSS. The media in the apical chamber was then

replaced with Insulin solution or 10 mg of P, PI or ILP was added to the existing HBSS in the apical chamber. TEER values were measured at 0, 0.5, 1, 1.5, 2 and 2.5-hour intervals. In addition, 100-200  $\mu$ l samples of apical and basolateral media were taken at each time point for insulin concentration determination. The medium in the apical and basolateral chambers were then removed and replaced with growth media and TEER was monitored further at 4, 8, 12 and 24 hours post-drugging. Samples of medium for insulin concentration determination were also taken at these times. For every four wells that were dosed, one well was used as a negative control and carried through the process with cells exposed only to pure HBSS and growth media respectively.

To further assess tight junction behavior, studies were also executed to determine the  $\text{Ca}^{2+}$  dependency of TEER. In this case, cells were either exposed to HBSS containing full calcium, 1.2 mM, or HBSS with minimum calcium, 0.2 mM instead of insulin or insulin/polymer samples. The data time scale and data collection procedure were analogous to the above.

#### **5.2.8 Insulin ELISA EIA**

Insulin concentration in the medium was determined using ELISA. Bovine Insulin EIA kits (Part # 008-10-1131-01) and Insulin Control Standards (Part # 008-1135-01) were obtained from ALPCO LTD, Windham, NH. Media/HBSS samples were prepared for assay via serial dilutions of 1:10, 1:100 and/or 1:1000. Typically, apical media samples were diluted 1:100 and 1:1000 and basolateral samples were diluted 1:10 and 1:100 based upon the expected concentration differences across the

cell monolayer in order to ensure the determined values fell well within the standard curve of the assay. These dilutions were carried out in 96-well plates on a Caliper Rapidplate 96/384 Workstation liquid handler (Caliper Life Sciences, Hopkinton, MA 01748), which also provided extensive mixing cycles between liquid movement steps. The cells were diluted with HBSS or cell media depending upon sample type.

Once the correct dilutions were obtained, an 80-well transfer was performed onto the ALPCO EIA plate. The remaining 16 wells were filled with insulin control standards of varying concentrations and known insulin ‘hi/lo’ controls, which were used in the post-assay data processing to establish a standard concentration curve. The assay was performed via manufacturer protocol and the plates were read on a Perkin Elmer Envision plate reader (Perkin Elmer, Downer’s Grove, IL 06484).

## **5.3 Results and Discussion**

### **5.3.1 Synthesis, characterization and insulin loading of hydrogel**

Prior studies in our lab have documented several key parameters of this system. The optimum particle diameter for transport has been determined to be  $\sim 40$   $\mu\text{m}$  and that particle size was used<sup>12</sup>. The pre-digested ILP were found to be 6.4% insulin by weight via loading and release study utilizing HPLC. As 10 mg samples were used for dosing, the maximum possible initial insulin load would be 450  $\mu\text{g/ml}$ .

### **5.3.2 Transepithelial electrical resistance and tight junction monitoring**

In an effort to characterize the specific mechanism of insulin transport as transcellular (across cells) or paracellular (between cells via tight junctions), the

transepithelial electrical resistance model (TEER) of tight junction integrity was employed. Figure 5.1 shows the changes in TEER over time for the varying undigested sample types. The negative control (C), I and ILP samples show negligible TEER changes over time whereas the P and PI samples demonstrate slightly higher TEER variations. All TEER values returned to normal within 24 hours of dosing. Similarly, there are only negligible TEER changes observed in the cases of SGF and SIF samples, as shown in Figure 5.2.

### 5.3.3 Insulin transport

The possible routes of drug transport across cells are depicted schematically in Figure 2.2 as adapted from Smith <sup>25</sup>. In order to qualify and quantify insulin transport, it is necessary to understand the initial concentration of the media in the apical chamber. Four apical starting conditions were studied and are shown in Figure 5.5. In the case of insulin only, an insulin solution was prepared in HBSS to a final concentration of 200  $\mu\text{g/ml}$ . This is the initial insulin concentration ( $C_{ia}$ ) for the 'I' and 'PI' samples. The PI samples also contained 10 mg of 'un-loaded' hydrogel particles suspended in an insulin solution of the same concentration, 200  $\mu\text{g/ml}$ . The initial apical media insulin concentration of the ILP, SGF and SIF samples ( $C_{ia}$ ) was zero, although insulin release began immediately upon contact of these sample types and the apical chamber media. At  $t=0$ , all samples were added to the apical chamber. In the case of ILP, SGF and SIF, 10 mg of particles were added to the apical chamber medium. In the case of I and PI, the apical medium was removed and replaced with insulin solution (I) and insulin solution plus 10 mg of unloaded polymer particles (PI). Insulin transport was quantified and is expressed as concentration profiles of the

apical and basolateral chambers in Figures 5.3 and 5.4 respectively. It should be noted that the apical concentrations presented in Figure 5.3 cover time points beginning at 4 hours, as the concentrations prior to that were known initial values. In the insulin samples, the concentration of the apical chamber remained relatively constant over time, during the 2.5 hour dosing, at  $\sim 200 \mu\text{g/ml}$  and the insulin transport across the cells was minimal. The apical concentrations decreased as expected after drug removal from  $200 \mu\text{g/ml}$  to  $\sim 25 \mu\text{g/ml}$ , as shown in Figure 13, reaching final basolateral concentrations of  $100 \text{ ng/ml}$  or less, as shown in Figure 14. In the case of insulin + polymer, the initial apical concentration dropped quickly from  $\sim 200 \mu\text{g/ml}$  to  $\sim 100 \mu\text{g/ml}$  during the dosing and stayed essentially constant in the apical chamber after dosing at  $\sim 100 \mu\text{g/ml}$  and reached final basolateral concentrations of  $\sim 100 \text{ ng/ml}$ . In the case of insulin-loaded-polymer (ILP) and ILP digested in simulated gastric fluid with pepsin (SGF), the apical concentrations rose rapidly from zero to  $\sim 300 \text{ ng/ml}$  during dosing and remained higher than the insulin alone and the polymer + insulin samples at  $\sim 300 \text{ ng/ml}$ . This is likely due to the incomplete removal of all polymer particles in the apical chamber when drug was removed due to adhesion of the ILP to the cell monolayer. In the cases of these ILP and SGF samples, peak basolateral concentrations of  $800 \text{ ng/ml}$  and  $600 \text{ ng/ml}$  respectively were reached at 8 hours post dosing. Finally, in the case of samples subjected to digestion in simulated intestinal fluid with pancreatin (SIF), the apical and basolateral insulin concentrations never rose above  $50 \text{ ng/ml} \pm 10 \text{ ng/ml}$ , apical data not shown. The differences in concentration profiles between the sample types can be explained by the schematics of Figures 5.5-5.8. Figure 5.6, demonstrates the

high and constant insulin concentration in the apical chamber. As very little insulin is transported across the cell monolayer, the apical insulin concentration remains essentially constant. In Figure 5.7, the same insulin solution is loaded in the presence of unloaded polymer and a significant decrease in apical insulin concentration is observed while there is little insulin transport across the cell monolayer. This is assumed to be due to uptake of insulin by the hydrogel system as expected at the pH of the apical media chamber. Figure 5.8 demonstrates the mass transfer model of the ILP, SGF and SIF samples. In the case of ILP and SGF, the initial insulin concentration in the apical chamber is low but steadily increase as the hydrogel matrix relaxes and releases insulin.

There is also significant insulin transfer across the cell monolayer. In the case of SIF, low insulin concentrations are observed in both the apical and basolateral chambers. This can be explained by the expected release of insulin from the loaded polymer samples during the SIF digestion, which is performed at near neutral pH. As there is little insulin left in the polymer post-SIF digestion, the release studies confirm low levels of insulin transport. Figure 5.4 demonstrates the concentration changes in the basolateral chamber. In the case of insulin only and polymer + insulin, the maximum concentrations of  $100 \pm 20$  ng/ml were obtained after 4 hours. In the cases of ILP and SGF, the maximum basolateral insulin concentrations of  $700 \text{ ng/ml} \pm 100$  ng/ml were obtained approximately 8 hours after dosing. Finally, the SIF samples demonstrated negligible insulin transport with the basolateral concentrations never exceeding 50 ng/ml. The results of the transport study are consistent with earlier work in a closed loop intestinal *in vivo* model <sup>12</sup>. This study demonstrated



bioavailability of 4.6%-7.4% from ILP dosing, whereas insulin alone provided significantly less than 1% bioavailability. In the current work, the ILP samples and SGF samples demonstrated

significantly higher insulin transport in the Caco-2 model than did insulin alone at a similar ratio of, approximately, 7:1 (700 ng/ml  $\pm$  100 ng/ml for ILP and SGF versus 100  $\pm$  20 ng/ml for insulin alone and polymer + insulin). Lastly, it should be noted that the release profiles of the ILP, SGF and SIF samples provided further evidence of the ability of this complexation hydrogel system to both ‘protect’ insulin through the low pH and enzymatic environment of the stomach and also to subsequently release biologically active insulin into the small intestine under the appropriate physiological conditions.

### 5.3.4 Permeability Quantitation

As an accurate comparison of the observed transport phenomena must include all aspects discussed above and drug permeability calculations ( $P_{app}$ ) are the most commonly accepted overall measure. Table 5.1 shows the permeability coefficients for all sample types as calculated using the specific form of Fick’s first law proposed by Youdim et al to enable mass balances (Eqn. 1),<sup>32</sup>.

$$P_{app} \text{ (cm/s)} = V_D / (A M_D) \times (\Delta M_R / \Delta t) \quad (\text{Eqn. 1})$$

Here  $V_D$  = the apical (donor) volume ( $\text{cm}^3$ ),  $M_D$  = apical (donor) amount (mol),  $\Delta M_R / \Delta t$  = change in amount of compound in the basolateral (receiver) chamber over time, and  $A$  = the membrane surface area ( $\text{cm}^2$ ). The permeability coefficients for each

sample type further highlight the differences in insulin transport between the sample types. The permeabilities for SGF and SIF are significantly greater than those for insulin and insulin in the presence of polymer, ~110-fold to ~180-fold. Lastly, as expected, the permeability for ILP digested in SIF is the lowest and is only a rough estimate as the apical and basolateral insulin concentrations were barely detectable.

### 5.3.5 Discussion

Earlier work has suggested that the insulin transport enhancing effects of this hydrogel system may be due to the ability of the system to bind free calcium, which can open tight junctions and facilitate insulin transport via the paracellular route<sup>18</sup>. In this study, the ILP and SGF sample types show the lowest variations in TEER but the highest amounts of insulin transfer. Assuming that TEER is an adequate quantitative measure of tight junction integrity, high insulin transfer rates in the absence of tight junction ‘loosening’ would suggest a transcellular or active transport mechanism. If this were the primary vehicle of insulin transport, significantly higher transport values would be expected in the case of the highest concentration gradient, the insulin only samples, but this is not observed. Similarly, the highest TEER fluctuations are seen in the case of PI samples, where the lowest transport is observed. The higher TEER fluctuations for these samples can be readily attributed to the surface chemistry of the PI samples. As the insulin loading process involves treating the insulin-saturated particles with 0.1 N HCl; to collapse the hydrogel network, polar surface groups on the gel surfaces are neutralized by the H<sup>+</sup> ions in this step. As the PI samples are never exposed to the acid treatment, the unreacted polar groups result

in microparticles that are slightly charged as detected by the TEER measurements. Lastly, it has been previously shown that this system is not cytotoxic, so cell death and/or damage may be ruled out as possible routes of insulin transport<sup>18</sup>. In light of these results, it is probable that the mechanism is mixed, i.e. elements of paracellular and transcellular transport are involved. As previously discussed, the Caco-2 cells do contain the insulin receptor so active transport cannot be ruled out. In addition, other factors may contribute such as polymer particle contact time and/or entrapment of the ILP polymer particles on and between the cell surfaces. In the case of ILP and or SGF where the most transport was observed, it may simply be that the loaded polymer seats itself nicely on the cells and provides a light and steady flow of insulin to the cell surfaces for transport. In the case of PI dosing, the unloaded polymer may be incorporating insulin into its matrix while simultaneously lowering the contact between insulin and the cells as the polymer may act as a barrier. In the case of insulin only, there may be inadequate contact of the insulin solution due to mucosa or other debris on the cell's surfaces, which can effectively 'block' the insulin receptors of the Caco-2 cells.

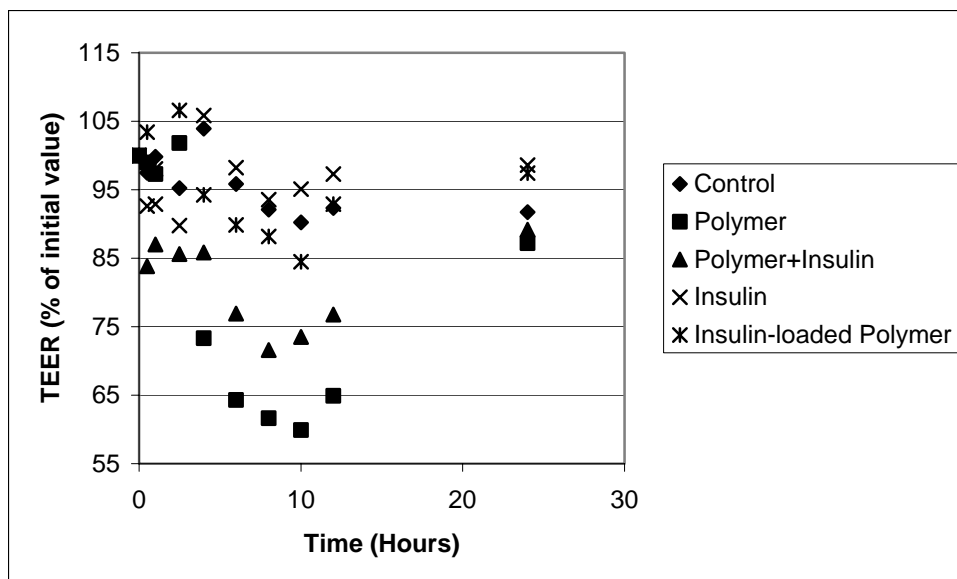
## 5.4 Conclusions

The ability of the novel poly[methacrylic acid-grafted-poly(ethylene glycol)] [P(MAA-g-EG)] hydrogel system to protect insulin through gastric conditions was confirmed via a dissolution study in simulated gastric fluid with pepsin. Similarly, the ability of this system to release active insulin was demonstrated via dissolution studies in simulated intestinal fluid with pancreatin. The previously demonstrated *in*

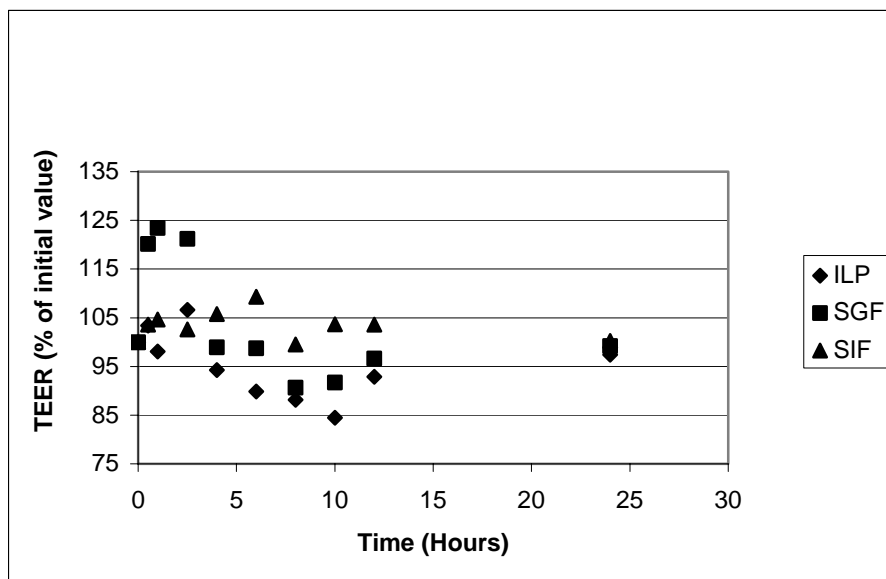
*in vivo* capability of this system to enhance insulin transport was confirmed *in vitro* using a Caco-2 cell model of intestinal absorption to study hydrogel samples that had been sequentially digested in simulated gastric and intestinal fluids containing proteolytic enzymes. The insulin loaded polymer (ILP) system, and the ILP subjected to digestion in simulated gastric fluid with pepsin, demonstrated permeability coefficients that were approximately 100 times greater than those of insulin alone. ILP, which had been subjected to digestion in simulated gastric fluid, demonstrated a minor increase in insulin transport analogous to that of undigested ILP. Transepithelial electrical resistance (TEER) studies were utilized to monitor tight junction integrity. As the highest rates of insulin transport did not correlate with the greatest variations in TEER, it is concluded that transport is most likely not due to the paracellular mechanism alone. Future pre-clinical *in vivo* and *in silico* studies will be carried out to determine the required effective dosing for insulin delivery using this system, the pharmacokinetics of the system and to determine the elimination properties.

**Table 5.1: Comparison of Permeability Coefficients (cm/s) and TEER Changes (% of initial value) in Insulin, Insulin + Polymer, Insulin-loaded Polymer (ILP), ILP Digested with SGF and ILP Digested with SGF and SIF.**

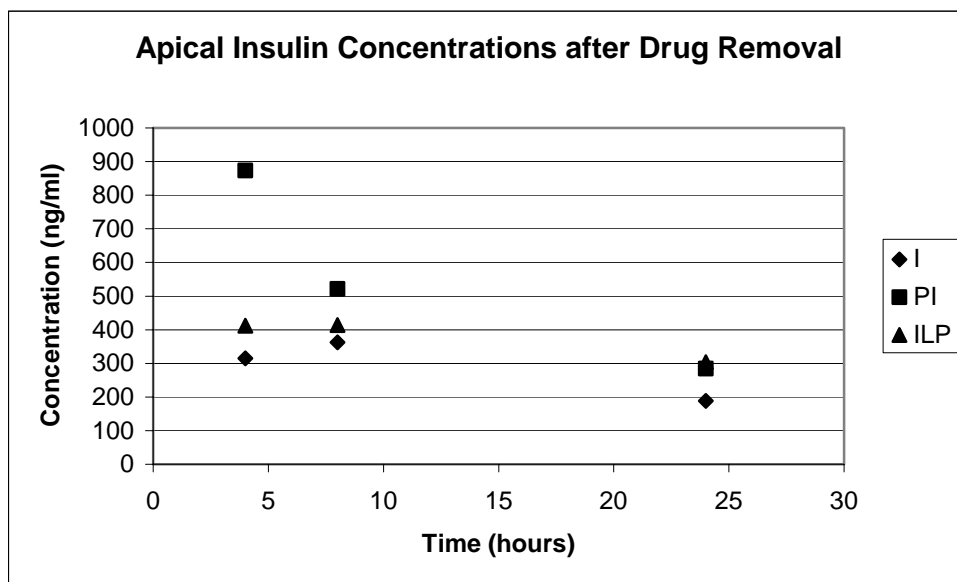
<b>Sample Type</b>	<b>Permeability (cm/s) (Papp x 10<sup>8</sup>)</b>	<b>Maximum TEER Change (% of initial value)</b>
<b>Insulin</b>	0.07	less than 10%
<b>Polymer+Insulin</b>	0.06	30 ± 10%
<b>Insulin-loaded polymer (ILP)</b>	12.7	16 ± 10%
<b>SGF-digested ILP</b>	6.61	less than 10%
<b>SGF and SIF digested ILP</b>	0.01	less than 10%



**Figure 5.1: Transepithelial electrical resistance trends during insulin transport for the negative control (untreated, C), polymer only (P), insulin only (I), insulin in presence of polymer (PI), insulin-loaded polymer (ILP)**

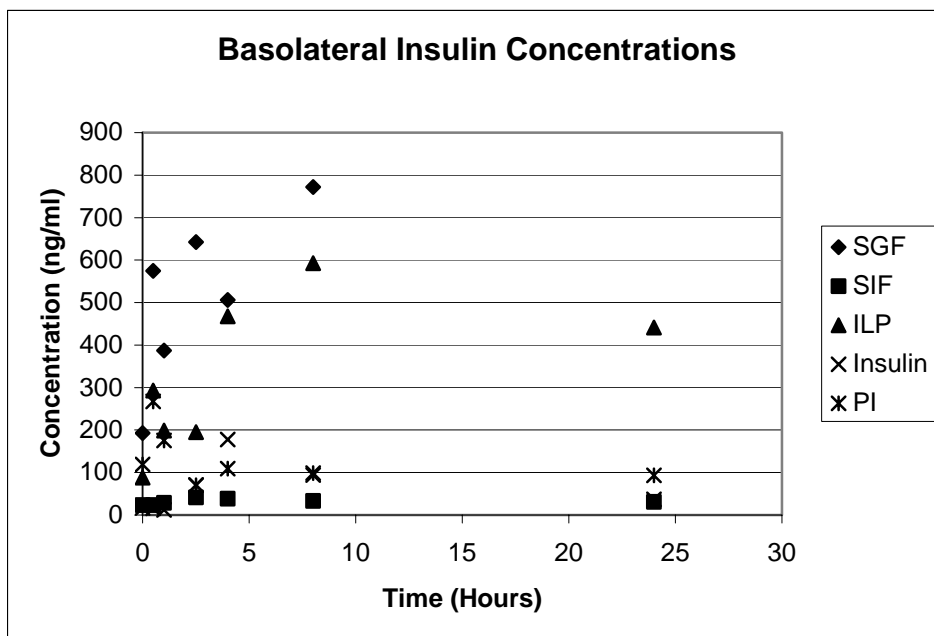


**Figure 5.2: Transepithelial electrical resistance trends during insulin transport for insulin-loaded polymer (ILP), ILP digested in simulated gastric fluid with pepsin (SGF) and SGF samples subjected to digestion in simulated intestinal fluid (USP) with pancreatin (SIF)**

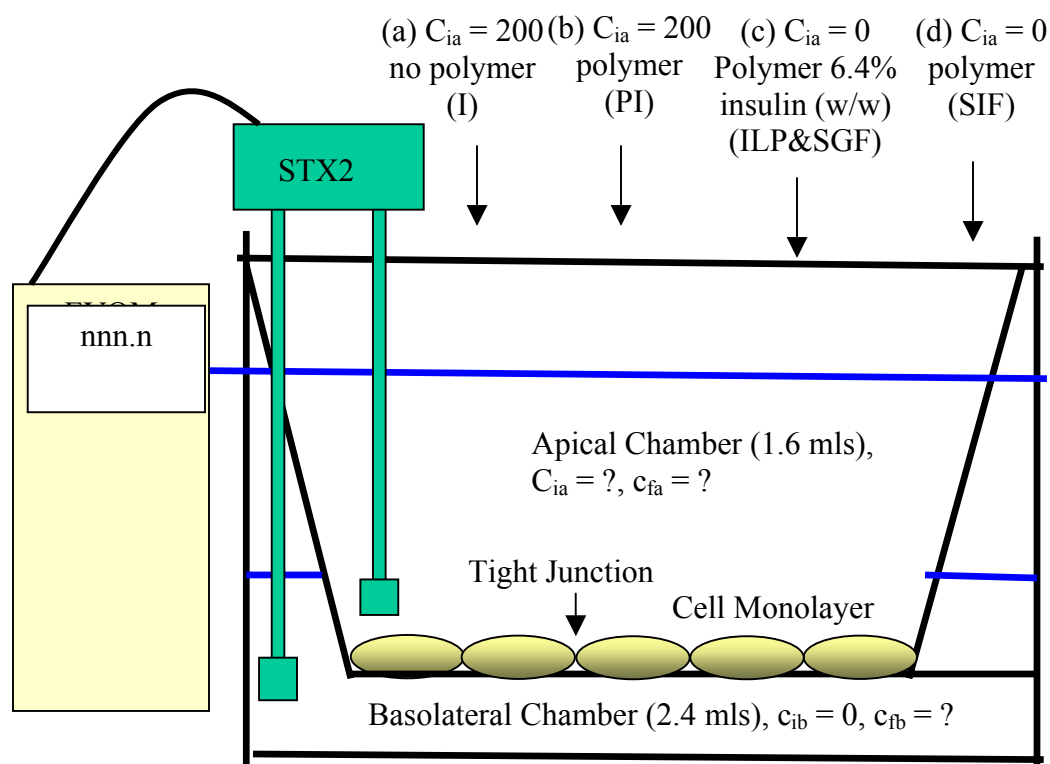


**Figure 5.3: Apical chamber insulin concentration for insulin only (I), insulin in presence of polymer (PI), insulin-loaded polymer (ILP)**

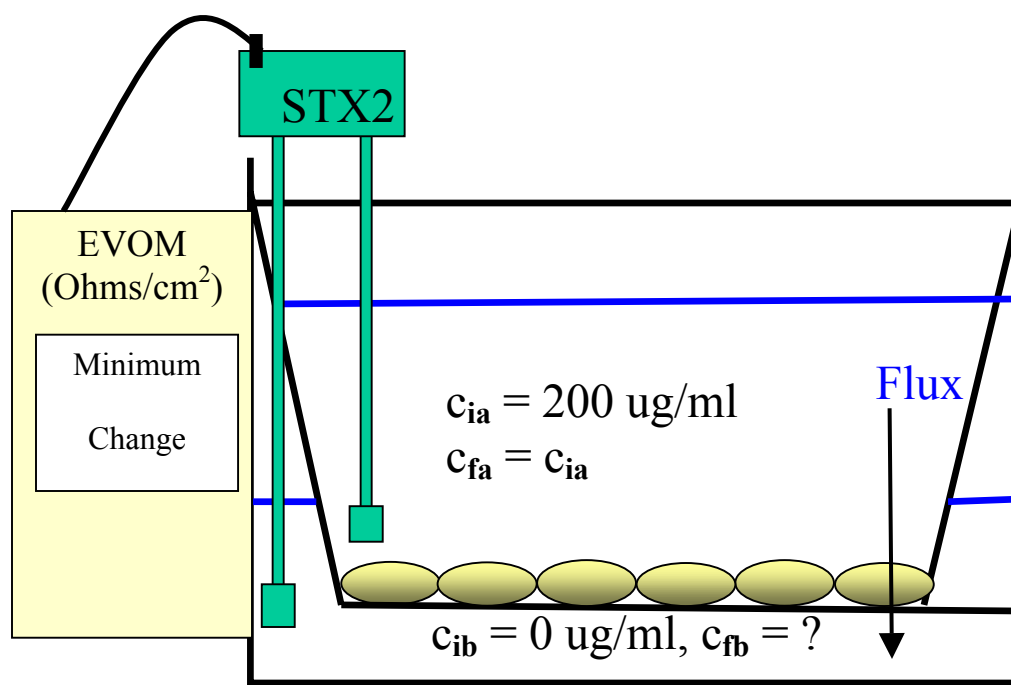




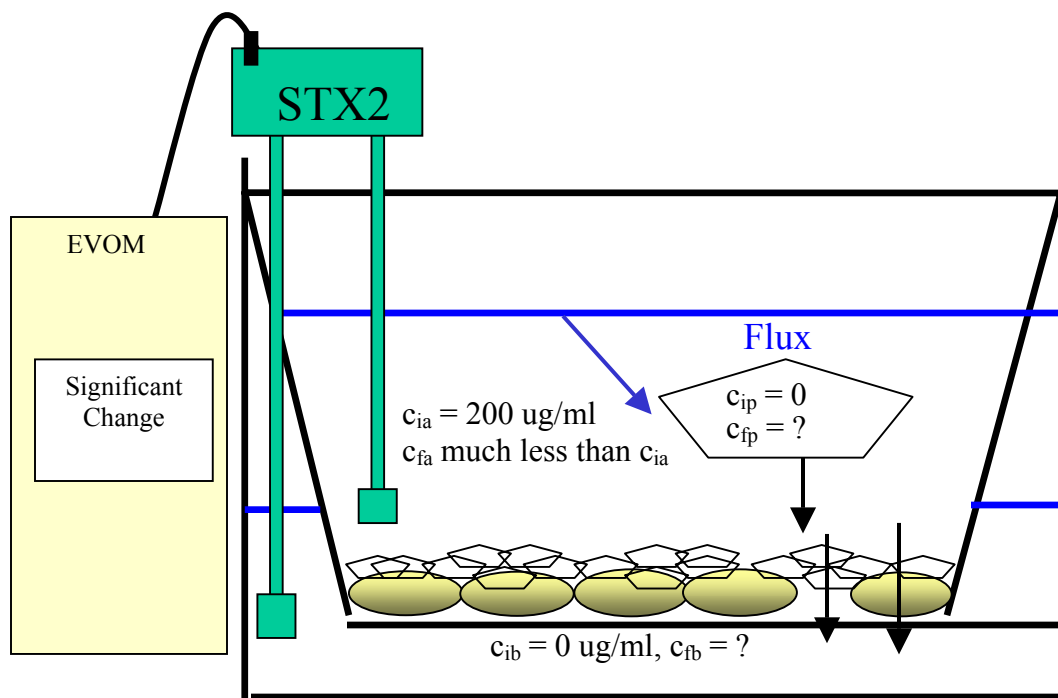
**Figure 5.4:** Basolateral chamber insulin concentration for insulin only (I), insulin in presence of polymer (PI), insulin-loaded polymer (ILP), ILP digested in simulated gastric fluid with pepsin (SGF) and SGF samples subjected to digestion in simulated intestinal fluid (USP) with pancreatin (SIF).



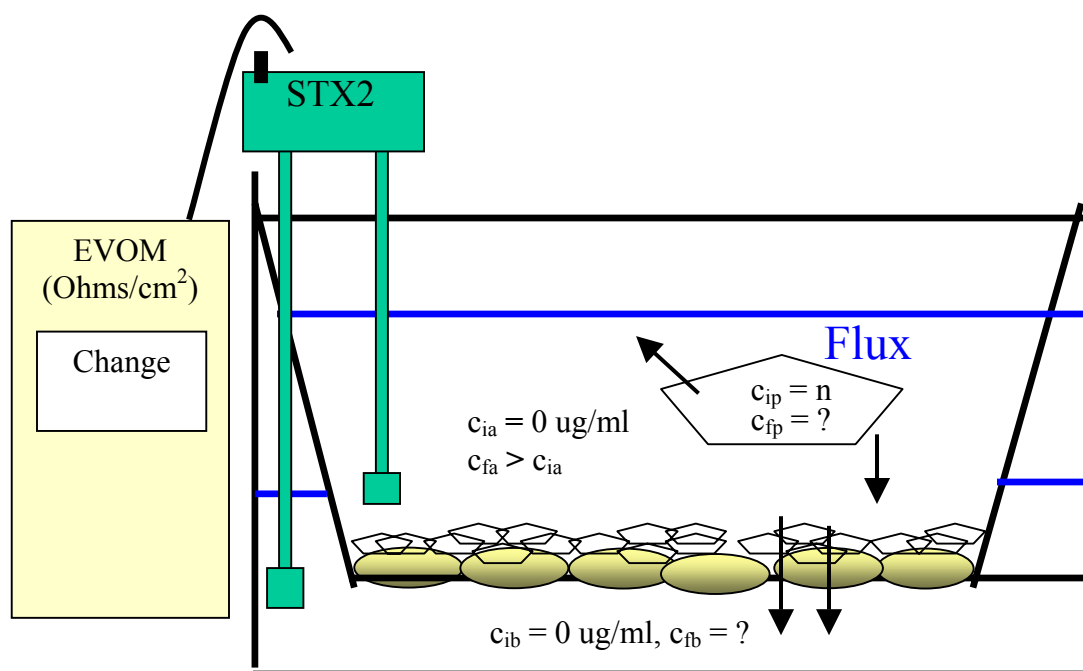
**Figure 5.5: Schematic of Experimental Apparatus and Description of Apical Starting Conditions.**  $C_{ia}$  and  $C_{fa}$  are the Initial and Final Concentrations in the Apical Chamber and  $C_{ib}$  and  $C_{fb}$  are the Initial and Final Concentrations in the Basolateral Chamber. (a) Insulin-only; (b) Polymer in Insulin Solution; (c) Insulin-loaded-polymer (ILP) and ILP subjected to Simulated Gastric Fluid with Pepsin; (d) ILP Subjected to Simulated Gastric Fluid with Pepsin and Simulated Intestinal Fluid with Pancreatin



**Figure 5.6: Schematic of Insulin Transport and Tight Junction Monitoring of Caco-2 Monolayers in the case of Insulin (I) Dosing**



**Figure 5.7: Schematic of Insulin Transport and Tight Junction Monitoring of Caco-2 Monolayers in the case of Polymer + Insulin (PI) Dosing**



**Figure 5.8: Schematic of Insulin Transport and Tight Junction Monitoring of Caco-2 Monolayers in the case of Insulin-loaded Polymer (ILP) Dosing**

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## CHAPTER 6: PHARMACOKINETICS AND PHARMACODYNAMICS OF NOVEL COMPLEXATION HYDROGELS FOR ORAL INSULIN DELIVERY IN RATS AND DOGS

### 6.1 Introduction

According to the Center for Disease Control, diabetes has become the fastest growing disease in the world. The consensus of the American Diabetes Association is that intensive insulin therapy associated with comprehensive self-management training should become the standard therapy in patients after puberty<sup>1</sup>. It is this comprehensive self-management requirement that brings the highest risk and burden. While there are four classes of oral anti-diabetic agents available for Type II diabetes, the standard of treatment for Type I is the subcutaneous injection of synthetic human insulin. Oral delivery of protein therapeutics, such as insulin, has the potential to increase patient compliance, lower medical treatment costs and provide superior pharmacokinetic profiles<sup>2</sup> but these benefits have not yet been realized in insulin therapy<sup>3,4</sup>.

In order to obtain adequate oral bioavailability of protein therapeutics, the protein must be protected from degradation from proteolytic enzymes as it passes through the upper gastrointestinal tract. Towards this goal, hydrogel technologies have been studied extensively<sup>5-11</sup> and a pH-responsive poly(methacrylic acid-g-ethylene glycol) system (P(MAA-g-EG)) has been developed and studied for the specific goal of oral insulin delivery.<sup>12-15</sup> Previous studies of this system have demonstrated the ability of this system to protect insulin through simulated gastric conditions<sup>16,17</sup> and to enhance insulin bioavailability in a closed loop *in vivo* model

Prior *in vivo* study of the P(MAA-g-EG) system demonstrated insulin bioavailability in *in situ* closed loop experiments on Sprague Dawley rats that exceeded treatment with insulin alone<sup>14</sup>. In these studies, closed sections of rat intestine were cleansed and dosed with insulin alone and with insulin-loaded polymer samples (ILP). The ILP samples produced relative systemic bioavailability values of 4.6 – 7.4% while that obtained from insulin alone was significantly less than 1%. While the specific mechanism by which this system enhances transport is unknown, a mixed mechanism that combines paracellular and transcellular transport is suspected based upon *in vitro* studies using Caco-2 cells<sup>16</sup>. Further, a relative bioavailability of 9.5% to subcutaneous insulin was claimed in a recent study of this hydrogel system in type 1 and type 2 diabetic rats<sup>18</sup>.

As all *in vivo* testing to date has resulted in determination of relative bioavailabilities, the aim of the present study is to determine the absolute oral bioavailability of insulin delivered via the P(MAA-g-EG) system in *in vivo* models that more closely mimic the intended clinical application of such a system. Pharmacokinetic and pharmacodynamic studies were performed in rats and dogs using iv/oral crossover studies.

Rats were dosed with human insulin intravenously at 1U/kg. At four-day intervals, the same animals were dosed with insulin via gavage with ILP at an oral dose of 50 IU/kg. The iv dose was selected based upon the clinical standard of 1 U/kg. Blood serum was collected for human insulin monitoring using an EIA assay that was specific for human insulin with minimal cross-activity with rat insulin.

The canine studies were conducted utilizing a modified glucose and insulin clamping technique as originally described by deFronzo et al <sup>19</sup>. Briefly, this technique involves the intravenous administration of basal levels of insulin and glucose to hold the blood glucose of the subject at a specified desired value as determined by frequent blood sampling. The test article is then administered and the glucose infusion rate is modified as needed to retain the targeted blood glucose value for a specified length of time. Although laborious, this technique offers many benefits including greatly increased safety against hypoglycemia and real time data and sample collection for detailed pharmacodynamic (glucose and insulin infusion rates, blood glucose, c-peptide) and pharmacokinetic (serum insulin) analysis.

## **6.2 Experimental Section**

### **6.2.1 Animals**

All studies were performed in compliance with the Organization for Economic Cooperation and Development principles of Good Laboratory Practice.

Nine female Sprague-Dawley rats (200 – 250 g) were obtained from Charles Rivers Laboratory, Portage, Michigan USA. The animals were maintained as per Centocor R&D Vivarium policy. Cage cards labeled with animal number, test article and IACUC protocol number (P-2005-462) and study number were affixed to the cages.

Four random mixed breed female dogs, each weighing approximately 18 kg, were obtained the Thomas Jefferson College of Medicine Vivarium. The dogs were kept in separate cages and fed Hills Science Diet dry and canned foods. Cage cards

labeled with animal number, test article and IACUC protocol number and study number were affixed to the cages.

### **6.2.2 Materials.**

For the rat studies, methacrylic acid (MAA) and insulin (human) were purchased from Sigma Aldrich (St. Louis, MO). Dimethoxy propyl acetophenone (DMPA) was purchased from Aldrich (Milwaukee, WI). The MAA was purified via packed column of DE-HIBIT 200 (Polysciences, Warrington, PA) in order to remove the inhibitor (hydroquinone monomethyl ether). Methoxy-terminated poly(ethylene glycol) monomethacrylate 1000 (PEGMA), and tetraethylene glycol dimethacrylate (TEGDMA) were all purchased from Polysciences, Warrington, PA.

For the canine studies, Novolin R, regular human insulin, was obtained from Novo Nordisk. Normal saline (0.9% NaCl in H<sub>2</sub>O) iv bags, 1 liter and 100ml, were obtained from Baxter Healthcare, Deerfield IL. One-liter Dextrose solution (20%) iv bags were obtained from Hospira Lake Forest, IL.

### **6.2.3 Polymer Preparation**

The polymer preparation was consistent across both species. Microparticles of P(MAA-g-EG) were prepared via free-radical solution photo-polymerization of MAA and PEGMA<sup>14</sup>. The monomers were mixed in appropriate molar ratios to yield a 1:1 ratio of MAA:EG units in the resulting hydrogel. The solution was then diluted to 44.7% (wt./wt.) of the total monomers with a 1:1 (vol./vol.) mixture of ethanol and water. TEGDMA was added as a cross-linking agent at  $X = 0.075$  moles

TEGDMA per MAA. DMPA was added as the initiator in the amount of 1% weight of the monomers. The reaction mixture was pipetted into a mold made of 2 glass plates held apart by 0.8mm spacers, creating a polymer film. The reaction was initiated by exposing the monomer film to UV light (Ultracure 100, Efes, Buffalo, NY) at  $1 \text{ mW/cm}^2$  at 365 nm for 30 minutes. The hydrogel films were removed from the molds, rinsed for 1 week in distilled  $\text{H}_2\text{O}$  (changed daily) to remove the unreacted monomers and sol fraction.

#### **6.2.4 Insulin Loading**

Hydrogel films were completely dried under vacuum at  $37^\circ\text{C}$ . The dried films were then pulverized into fine particles using a mill (Bell Art Products, Pequannock, NJ). The particles were further ground using a mortar and pestle and then passed through sieves with a mesh size of  $43 \mu\text{m}$ . Insulin loading was achieved by dissolving 10 mg of human insulin in 20 ml of PBS ( $\text{pH} = 7.4$ ). The crushed polymer microparticles were dispersed in the insulin solution at  $37^\circ\text{C}$  and stirred at 300 rpm. After 6 hours, a 0.2ml aliquot was removed via syringes equipped with  $0.45 \mu\text{m}$  membrane filters and 10 ml of 0.1N HCl was added to the solutions to collapse the polymers. The resulting solutions were filtered with cellulose acetate/cellulose nitrate filters ( $0.45 \mu\text{m}$ , Fisher Scientific, PA). The polymers were further washed with 100 ml 0.1N HCl and 100 ml of de-ionized water. All glassware was siliconized via treatment with Sigmacote® (Sigma). The resulting insulin-loaded polymer (ILP) was dried in a lyophilizer (Virts, Gardiner, NY) at  $-80^\circ\text{C}$  and stored at

-20°C. HPLC was used to determine the insulin content of the wash solutions and the resulting insulin content of the ILP was determined via a simple mass balance.

The above procedure was repeated to produce the negative control sample ‘dummy-loaded-polymer’, DLP. For these samples the above procedure was used except the microparticles were dispersed in a solution of PBS, instead of human insulin. All other steps were consistent with those above to ensure comparability.

### **6.2.5 Insulin Release**

To quantify the ability of ILP to release insulin, 10 mg ILP samples were dispersed via stirring at 300 rpm in 20 ml PBS in a siliconized vessel at 37 °C. Aliquots of 0.2 ml were taken via filter-equipped syringe at set time intervals. The volume was kept constant via immediate replenishment with equal volume of warm PBS. The insulin concentrations were determined via HPLC.

### **6.2.6 HPLC**

A Waters Symmetry® 300 column was used with a gradient of 25:75 acetonitrile/water to 45:55, including 0.1% TFA, over 20 minutes employed as the mobile phase. Samples were injected into a Waters 2690 separations module equipped with a 996 Photodiode Array detector at a 1ml/min flow rate.

### **6.2.7 Rat IV Crossover Study.**

On day 0, the study animals (n=3/ group) were assigned to 1 of 3 treatment groups, Control, Insulin and ILP. The animals in Group 1 received an iv injection of

0.25 ml of PBS. The animals in Groups 2 and 3 received an iv injection of 0.25 ml of human insulin (1 U/ml). All animals were bled at 0, 15, 30, 45, 60, 120 and 240 minutes post dosing for blood glucose and insulin EIA samples. The blood samples were collected via retro orbital sinus under CO<sub>2</sub> anesthesia, target volume 250 µl/bleed into 0.7 ml BD Microtainer serum separation tubes.

On Day 3, all animals were orally dosed. The Control animals received 1.0 ml of DLP (0 mg/kg insulin). The ILP animals received 1.0 ml ILP solution (50 IU/kg insulin) and the Insulin animals received 0.25 ml Insulin solution (1.0 U/ml). Blood samples were collected at 1, 2, 3 and 4 hours for human insulin EIA and blood glucose measurements. On day 7, the procedure was repeated exactly except the ILP dose was doubled to 100 IU/kg HI and the total volumes of the ILP and DLP doses were 2.0 ml. On day 10, the procedure was executed again as per day 7 except the ILP dose was escalated to 200 IU/kg HI. On days 7 and 10, blood samples were collected at 1, 2, 3 and 4 hours as per the day 3 protocol. The animals were fasted overnight only prior to each treatment and were allowed water ad libitum throughout the experiments.

#### **6.2.8 Human Insulin Measurement.**

The blood samples were allowed to clot at room temperature for 30 minutes in the separation tubes. Serum was obtained from the clotted samples via centrifugation at 2000 – 3000 g for 15 minutes at 4<sup>0</sup> C. The human insulin measurements were performed using the Linco Human Insulin ELISA kits (Linco Research, St. Charles, Mo 63304) Cat.# EZHI-14K, as prescribed by the manufacturer. This kit uses a

sandwich ELISA principle and is capable of specifically detecting 2 to 200  $\mu\text{U/ml}$  human insulin from a 20  $\mu\text{l}$  sample while any rat insulin is not detectable at concentrations below 120 nM. No dilutions were required in the rat study and all assays yielded results that were well within the quality tolerances of the manufacturer.

#### **6.2.9 Blood Glucose Measurements**

Blood glucose measurements were obtained using a OneTouch Ultra blood glucose meter and OneTouch Ultra glucose test strips (LifeScan Inc.).

#### **6.2.10 Data Processing and Analysis**

All samples were tested in duplicate in the ELISA plates. The absorbance values were measured at 450 nm using a Perkin Elmer Envision plate reader (Perkin Elmer, Downer's Grove, IL 06484). The dose response curve was constructed using the 4-parameter logistic equation functionality of GraphPad PRISM software, version 4.0 (GraphPad Software Inc.).

#### **6.2.11 Canine IV Crossover Study.**

Due to the laborious nature of the glucose clamping technique, only one animal can be treated per day and each treatment consists of only one dose. After being fasted overnight, the subject dog was transported from the Vivarium to the treatment suite. Intravenous catheters were inserted into each foreleg using 22 gauge BD Insite Autoguard shielded iv catheters. The dog was placed in a padded bed on heating pads set at a gentle level and one person was on the floor with the dog at all



times to monitor for stress and offer comfort. At time  $t=0$ , an initial blood glucose measurement was taken and the dog was connected to an iv infusion of 20% dextrose solution. Once the blood glucose reached 100 mg/dl, an iv infusion of insulin solution, 1IU/ml, was initiated. The initial infusion rates were set based upon literature values, primarily from veterinary literature and human glucose clamping protocols<sup>20-22</sup>. The infusions were accomplished using Alaris® IVAC Signature Gold Infusion pumps (Cardinal Health, Dublin, Ohio 43017) and the corresponding disposable iv cassettes and tubing sets. Blood sampling occurred every 5 minutes and the glucose infusion rate was titrated to ensure that the blood glucose remained steady at 100 mg/dl. Once the blood glucose remained between 90 and 110 mg/dl for more than 30 minutes, the clamp was established and a dose of test article could be given.

The test articles included a 'no sample' study, which was simply a control experiment of the clamping technique in each dog, an iv insulin bolus of between 0.1 and 0.2 IU/kg, oral ILP doses of 50 – 100 IU/kg, and an oral insulin solution of 0.1 – 0.2 IU/kg. The clamp was maintained and monitored for four hours post-dosing for all sample types. During this time, blood glucose measurements were taken every 5 minutes and serum samples were collected every 10 minutes for insulin and c-peptide quantification. In order to ensure the safety of the animal immediately after the procedure, the animal was slowly released from the clamp during the last of the four-hour experiment as follows. The insulin infusion was stopped at  $t = 3$  hours and 15 minutes post-dosing. The glucose infusion was terminated fifteen minutes later and the dog was fed and allowed to roam the treatment room. After 30 more minutes of sample collection and feeding, the dog was returned to her run. Each dog was

accessed a total of 4 times for treatment with at least 7 days rest between each treatment. After protocol completion, the dogs were successfully placed in loving homes.

### 6.2.12 Analytics

The blood glucose and human insulin analysis and data processing were conducted exactly as they were for the rat study using the Linco Human Insulin EIA, OneTouch blood glucose meter and the GraphPad Prism software respectively.

The canine c-peptide concentrations were determined using the Linco Canine C-Peptide RIA kits (CCP-24HK, Linco Research, St. Charles, Missouri 63304) as per the manufacturers instructions. The standard curve and data processing was accomplished using GraphPad Prism as per the EIA experiments above.

## 6.3 Results

The resulting dose response curve in the rat study from the iv dose is shown in Figure 6.1. The 2-hour and 4-hour time points have been removed to aid visualization of numeric integration of the iv curves shown in Figure 6.1 and the oral dose response curves, and the oral dose response curves, data not shown, over the entire 4-hour sampling time. The absolute bioavailability of insulin delivered from the hydrogel system was then determined as per Equation 1<sup>23</sup>. The area under the curve (AUC) values were determined by the method of least squares and utilized to determine bioavailability as shown in equation 6.1 below

$$\text{Bioavailability (F)} = \frac{AUC_{po}}{AUC_{iv}} \times \frac{Dose_{iv}}{Dose_{po}} \quad (6.1)$$

It was determined that the mean absolute bioavailability in rats of insulin delivered from this hydrogel system was approximately  $1\% \pm 60\%$ . The wide variation is due to significant differences in the results obtained from the 3 rats in the oral ILP group, which had individual bioavailability values of 0.2%, 0.1% and 2.5% respectively.

The canine study yielded similar results. Figure 6.2 demonstrates the basal insulin and glucose levels during a glucose clamp control experiment where no test article was given. After an initial blood glucose measurement at  $t=0$ , the animal was placed on an infusion of 20% dextrose until a blood glucose level of 100 mg/dl was obtained, which occurred at approximately  $t = 10$  min. At that time, an infusion of human insulin is initiated at a steady rate (IIR). The glucose infusion rate (GIR) was varied to 'clamp' the blood glucose at 100 mg/dl. After  $\sim 3.0$  hours, the insulin infusion was discontinued followed by the cessation of the glucose infusion. The animal was fed and its blood glucose is monitored for an additional 30 minutes to ensure safety. Figure 6.2 shows that the mean blood glucose level was successfully maintained at  $\sim 100$  mg/dl and the basal serum insulin concentration was  $\sim 10$  mU/l following the initial iv insulin bolus.

The details of a glucose clamp experiment containing an oral ILP dose is presented in Figure 6.3. This chart chronicles the entire 5-hour experiment. The initial upswing in the glucose infusion rates correlates with the administration of iv glucose followed by the iv insulin infusion. By 50 minutes, the blood glucose and glucose infusion rates have stabilized and basal insulin level has balanced at 20 mU/l and the oral ILP dose is administered.

Approximately 50 minutes later, the pharmacodynamic effect is most pronounced as demonstrated by the sharp increase in glucose infusion required to maintain the glucose clamp at 100 mg/dl. While the spike in serum insulin concentration lasts for just a few half-lives, the resulting pharmacodynamic effect last more than 80 minutes as evidenced by the duration of time the glucose infusion rates must be maintained above the basal values of 20-30 ml/hr. The insulin dose response curves for the iv and oral ILP doses for all four dogs are shown in Figures 6.4 and 6.5 respectively. As per the rat study, the curves were shifted so that the exact time of dosing, usually 60-90 minutes after initiation of the glucose clamp, was set equal to zero and integrated over a four-hour period to determine the AUC for each animal from each dose. The resulting mean absolute bioavailability was  $0.002 \pm 11\%$ .

The pharmacodynamic response to the ILP doses is presented in Figure 6.6. The clear and significant blood glucose lowering effects of the ILP doses are evident by study of the glucose infusion rates. In the case of 'Tawny', it is theorized that one of the two ILP capsules did not leave the stomach until much later in the experiment, resulting in the second glucose infusion rate peak. When normalized back to the time of dosing, the peak pharmacodynamic effect occurs between 50 and 90 minutes post oral dose.

In an attempt to model the fate of insulin delivered from the ILP system, the following correlations were applied <sup>24</sup>:

$$F_{\text{oral}} = (1-f_G)(1-f_H)(1-f_{\text{abs}}) \quad (\text{Eqn. 2})$$

$$f_G = 1 - \text{AUC}_{\text{po}}/\text{AUC}_{\text{hpv}} \quad (\text{Eqn. 3})$$

$$f_H = 1 - \text{AUC}_{\text{hpv}}/\text{AUC}_{\text{iv}} \quad (\text{Eqn. 4})$$

where  $f_{\text{abs}}$ ,  $f_{\text{G}}$ , and  $f_{\text{H}}$  represent the fraction of insulin lost in the gastrointestinal tract, at the gut wall and in the liver respectively. It is important to note that these equations are based on hepatic portal vein cannulation and  $\text{AUC}_{\text{hpv}}$  cannot be experimentally determined otherwise. The detailed pharmacokinetic results for both species are presented in Table 6.1. To ensure animal safety and to minimize morbidity, literature values for  $\text{AUC}_{\text{hpv}}$  were obtained and utilized <sup>25</sup>. The results of the application of the above model are presented in Table 6.2. As evidenced from the consistently high  $f_{\text{G}}$  values, this model suggests that the majority of insulin is lost at the gut wall. Lastly, it is clear from this data that the absolute oral bioavailability in rats and dogs is extremely similar if the single instance of  $F > 1\%$  is eliminated from consideration.

## 6.4 Discussion

Prior work on the oral insulin delivery capability of P(MAA-g-EG) hydrogels has reported bioavailability values that are significantly higher than those produced by this study. As previously discussed, Nakamura et al reported relative bioavailability amounts of 4.6% - 7.2% in *in situ* closed loop studies in rats <sup>14</sup>. Interesting results are obtained when the when the bioavailability model above is applied. In simulating the closed loop model,  $f_{\text{abs}}$  can be assumed to be zero as the ILP is placed directly into the loop of intestine and  $f_{\text{H}}$  can be assumed to be 0.5 from the literature. When the mean relative bioavailability from this study, 5.9%, is inserted into the model, the resulting value for the fraction of insulin lost at the gut wall ( $f_{\text{G}}$ ) is 0.882. As the experimental procedure included fasting the rats for 24 hours and the rinsing of the isolated ileal segment with a significant volume of

phosphate buffered saline (PBS), it is fair to assume that the difference in bioavailability between the current work and the *in situ* work lies in the treatment of the gut wall which results in a significantly lowered  $f_G$ , from 0.981 to 0.882 respectively. Specifically, in a fasted state, the enzymatic composition and action of the intestinal segment is expected to be greatly reduced<sup>26</sup>. Further, the PBS wash most likely decreased or altered the enzymatic composition of the glycocalix as closed loop studies, by the same authors, have demonstrated that PBS rinsing can lower enzymatic activity in rats<sup>27</sup>. This effect has been confirmed in unpublished work in our lab. That said, it is clear that the gut wall is the controlling resistance in both studies. Similarly, the variance in results between the current work and the recently published rat *in vivo* work can be explained. Morishita et al report a maximum relative bioavailability of 9.5% in their recent work but also report that the hypoglycemic effects of the ILP system decrease with shortening the fasted period<sup>18</sup>. As the rats in that study were fasted for 48 hours prior to the study and the rats in the current work were fasted overnight only, it is fair to surmise that some of the difference in experimental outcomes are due to the differences in fasting times. This hypothesis is further supported by basic physiology of the small intestine as it is well known that motility patterns of the small intestine are profoundly altered by eating and that gastric and intestinal secretions are greatly induced by feeding<sup>28</sup>. Based on these core concepts as well as the correlation of values obtained by the study of two species, the authors feel that the current work offers the most physiologically relevant model, presented to date, of the bioavailability of insulin delivered from this novel complexation hydrogel system.

As the low absolute bioavailability values reflect significant insulin loss, the authors have applied well-established kinetic models to determine the most likely points of loss as shown in Figure 6.6. It is clear that the loss at the gut wall ( $f_G$ ) is most significant in all cases but the loss in the gastrointestinal tract appears to vary significantly. This most likely due to the nature of mass transport and mixing in the lumen of the gastrointestinal system, which is often mathematically characterized via the principle of random walks<sup>29</sup>. The 1<sup>st</sup>-pass hepatic effect can be seen as negligible as it appears that most insulin has been lost prior to reaching the hepatic portal vein.

## 6.5 Conclusions

With respect to future work, this study provides clear insight and direction. First, it is clear that affecting insulin absorption at the gut wall can make the largest gains in oral bioavailability. Improving the mucoadhesive properties of the polymer system to prolong contact time with the gut wall as well as use of penetration/absorption enhancers may all offer benefits. Further, the current work demonstrates that the system does protect the protein from proteolytic damage in the upper gastrointestinal tract by design and that losses in the lumen of the small intestine are of smaller consequence. Lastly, as it is clear that the protein is arriving in the small intestine intact and is quickly released at the corresponding pH levels, it may make sense to consider the utilization of this system to deliver protein and peptide therapeutics that are intended to have regional effect versus systemic. One example would be the delivery of antibody or other protein therapies to the intestinal tract to treat local disorders such as Crohn's disease.

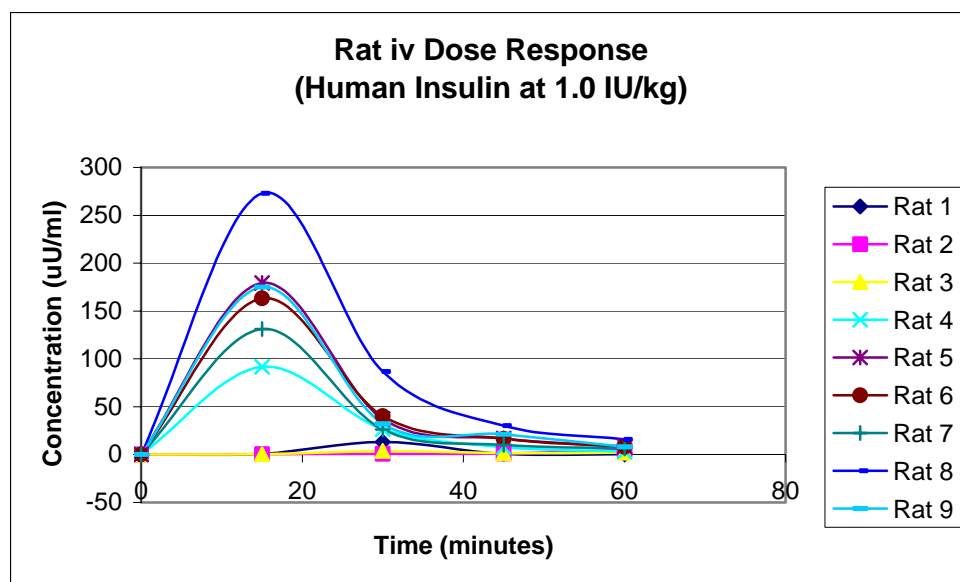
**Table 6.1: Absolute Bioavailability and Pharmacokinetic Parameters of Insulin Delivered via ILP**

<b>Dog</b>	<b>Dose<sub>iv</sub></b>	<b>Dose<sub>po</sub></b>	<b>AUC<sub>iv</sub></b>	<b>AUC<sub>po</sub></b>	<b>F<sub>Oral</sub></b>
Freckles	0.15	50	7605	3504	0.0014
Breezy	0.1	50	6321	6871	0.0018
Elvis	0.2	50	4369	7939	0.0022
Tawny	0.15	50	9333	4265	0.0014
Mean :	0.15	50	6907	5645	0.0017
Stdev:	0.040825	0	2094	2102	0.0004
<b>Rat</b>	<b>Dose<sub>iv</sub></b>	<b>Dose<sub>po</sub></b>	<b>AUC<sub>iv</sub></b>	<b>AUC<sub>po</sub></b>	<b>F<sub>Oral</sub></b>
Rat 1	1	50	7656	786.4	0.002
Rat 2	1	50	14246	750.7	0.0011
Rat 3	1	50	13450	17079	0.0254
Mean:	1	50	11784	6205	0.0095
Stdev:	0	0	3597	9417	0.014



**Table 6.2: Pharmacokinetic Parameters from Model of Insulin Loss**

<b>Dog</b>	<b>F<sub>h</sub></b>	<b>F<sub>g</sub></b>	<b>F<sub>abs</sub></b>	<b>F<sub>Oral</sub></b>
<b>Freckles</b>	0.42	0.998	0.0001	0.0014
<b>Breezy</b>	0.42	0.996	0.172	0.0018
<b>Elvis</b>	0.42	0.987	0.697	0.0022
<b>Tawny</b>	0.42	0.998	0.0001	0.0014
<b>Mean :</b>	0.42	0.994	0.290	0.002
<b>Stdev:</b>	0	0.006	0.371	0.0004
<b>Rat</b>	<b>F<sub>h</sub></b>	<b>F<sub>g</sub></b>	<b>F<sub>abs</sub></b>	<b>F<sub>Oral</sub></b>
<b>Rat 1</b>	0.5	0.998	0.0001	0.0014
<b>Rat 2</b>	0.5	0.998	0.999	0.0011
<b>Rat 3</b>	0.5	0.949	0.999	0.0254
<b>Mean:</b>	0.5	0.981	0.991	0.01
<b>Stdev:</b>	0	0.028	0.015	0.014



**Figure 6.1: Human Insulin iv Dose Response Curve in Sprague-Dawley Rats**

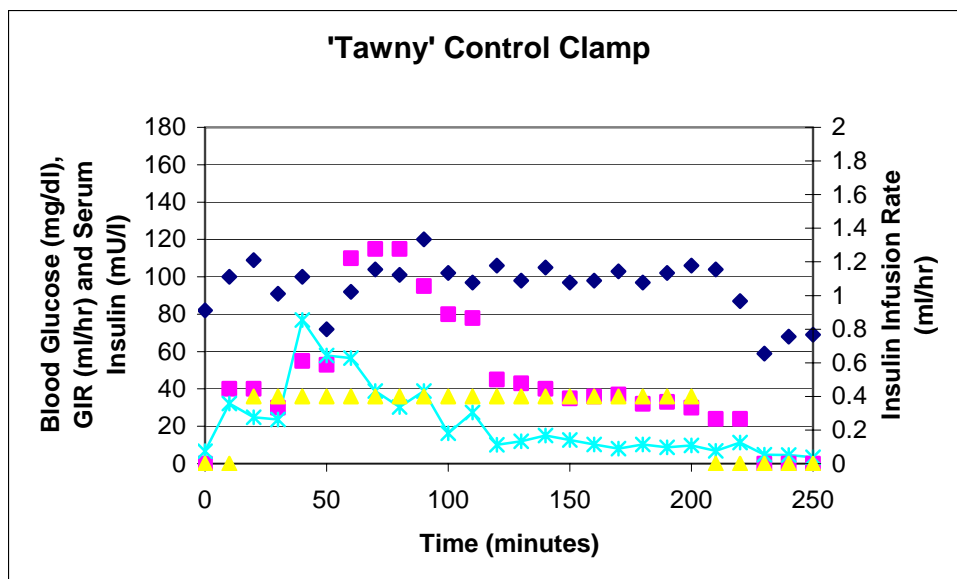
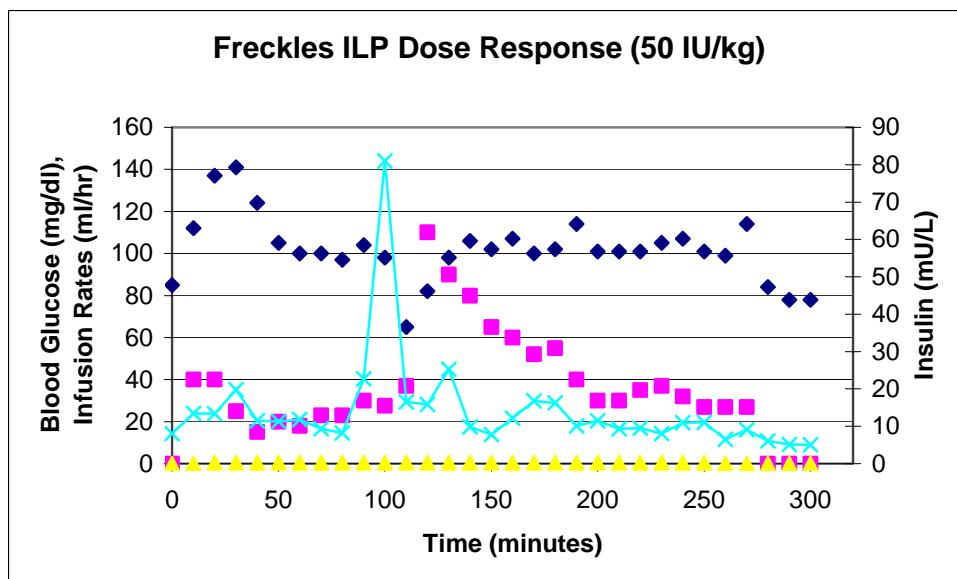
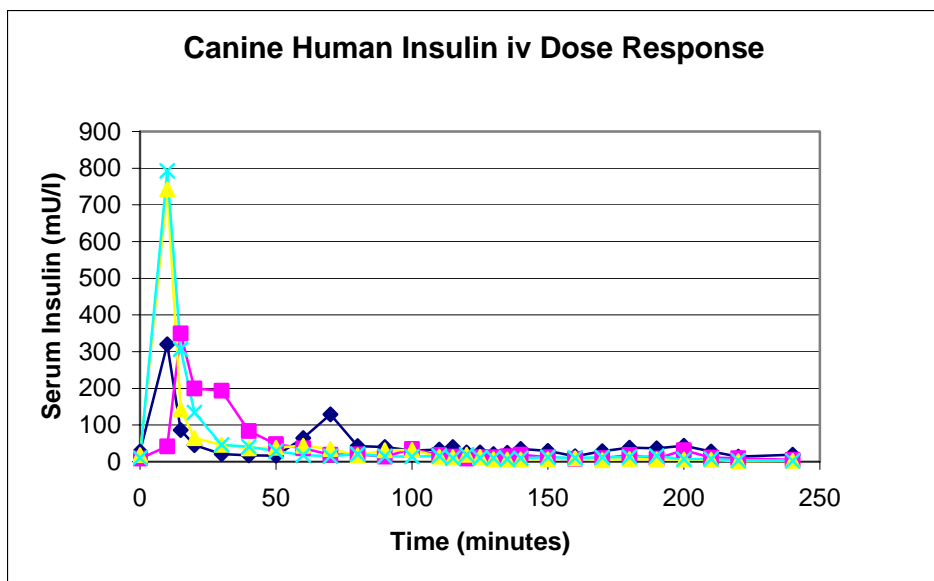


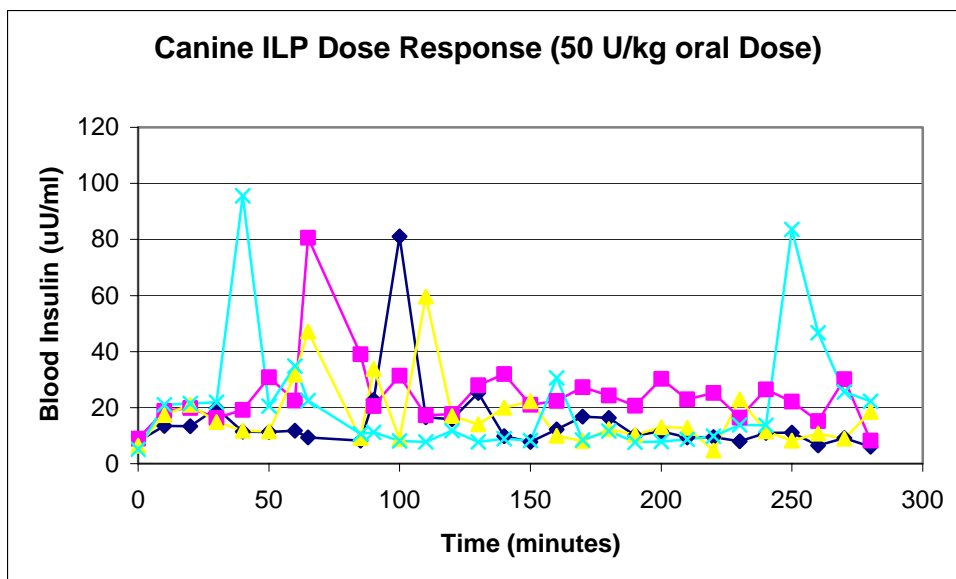
Figure 6.2: Euglycemic insulinemic clamp with no sample administered. ◆ Blood Glucose, ■ Glucose Infusion Rate, ▲ Insulin Infusion Rate, ★ Serum Insulin



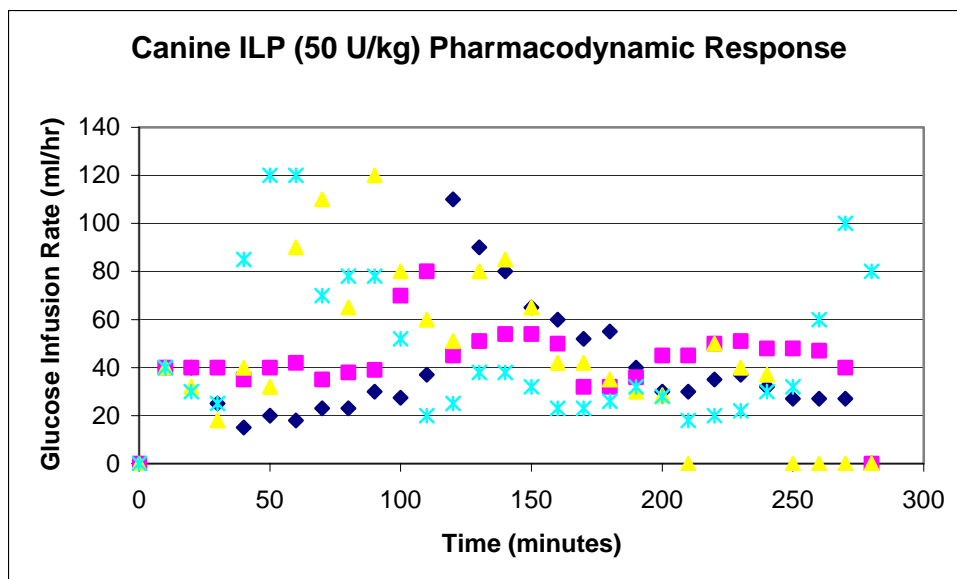
**Figure 6.3: Details of single ILP administration procedure. ◆ Blood Glucose, ■ Glucose Infusion Rate, ▲ Insulin Infusion Rate, ★ Serum Insulin**



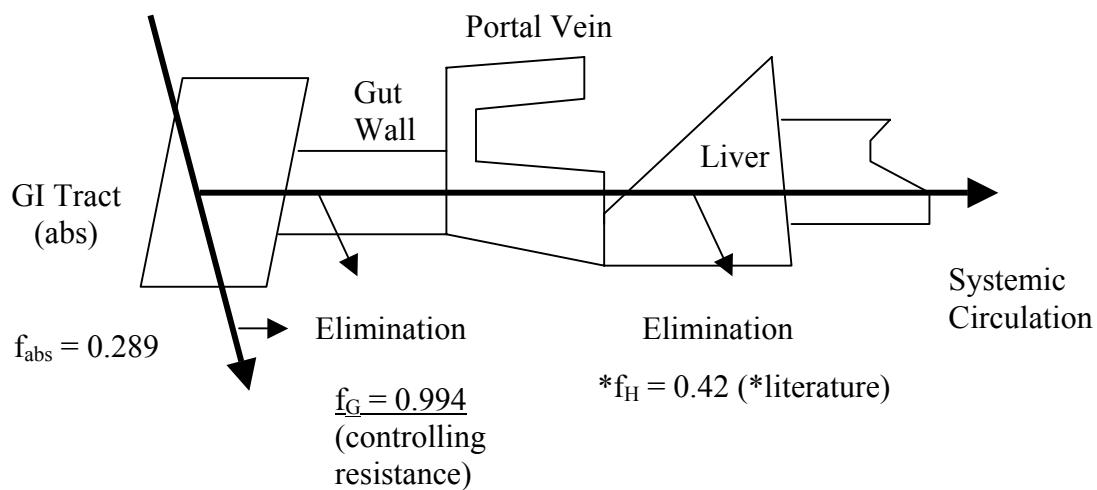
**Figure 6.4: Human Insulin IV Dose Response** ◆ ‘Breezy’ 0.1 U/kg, ■ ‘Freckles’ 0.15 U/kg, ▲ ‘Elvis’ 0.2 U/kg and ★ Tawny 0.15 U/kg



**Figure 6.5: Human Insulin ILP Dose Response** ◆ ‘Freckles’, ■ ‘Breezy’ ▲ ‘Elvis’ ★ Tawny



**Figure 6.6: ILP Pharmacodynamic Dose Response** ◆ ‘Freckles’, ■ ‘Breezy’  
 ▲ ‘Elvis, ★ Tawny



**Figure 6.7: Sites of insulin loss in the gastrointestinal system. Most insulin is lost at the gut wall where  $f_G = 0.994$ .**



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## **CHAPTER 7: INSULIN SATURABILITY. A CASE REPORT OF A MASSIVE INTRAVENOUS INSULIN OVERDOSE IN A HEALTHY DOG**

### **7.1 Introduction**

The current theory on insulin saturation assumes that there are two potential sites; the first site involves transporting the insulin across the endothelium, from the plasma to the interstitial fluid. The second site involves insulin binding to its receptors on insulin-sensitive cells and its downstream effects. In the current study, due to an administration error, an 18 kg dog received 180 units of insulin iv (10 IU/kg) instead of 1.8 IU total (0.1 IU/kg). The mistake was immediately realized and, due to the inherent safety-centric design of the euglycemic-hyperinsulinemic clamping approach, the dog successfully recovered after more than 10 total hours of treatment. During this time, blood glucose was measured every 5 minutes and serum was collected for serum insulin and c-peptide measurements every 10 minutes. The resulting data provide a truly unique view of insulin absorption, metabolism and elimination of intravenous insulin.

### **7.2 Experimental Section**

The original study design included the use of euglycemic insulinemic clamping in order to assess the absolute bioavailability of insulin delivered orally via a pH sensitive complexation hydrogel delivery system. Four random mixed-breed female dogs, each weighing approximately 18 kg, were obtained from the Thomas Jefferson University College of Medicine Vivarium for the study. After 4-5 days of acclimation in the vivarium, the dogs were to be accessed weekly for a single dose

response study for a total of 4-6 total treatments over 4-6 weeks. The study utilized a crossover design with a baseline clamp performed week 1, an iv insulin dose week 2, an oral insulin loaded polymer (ILP) dose week three and an oral insulin solution dose on week four. Weeks five and six were reserved for repeat dosing for any problem experiments and/or additional ILP administration at different doses.

### **7.2.1 Glucose Clamping Technique**

A euglycemic-hyperinsulinemic clamp was established based upon the methods of DeFronzo et al <sup>1</sup>. Briefly, the dog was fasted overnight and allowed water ad libitum. Intravenous catheters were inserted into each foreleg, one to be used for sample extraction and the other for intravenous administration. After an obtaining an initial, t=0, blood sample of approximately 0.6 mls, iv infusion of glucose was initiated at a rate of 133 mg/min dextrose. Blood glucose (bg) samples were drawn at 5- minute intervals and monitored using a OneTouch Ultra© meter until the blood glucose exceeded 100 mg/dl. Once this level was obtained, an insulin infusion was initiated at a rate of 0.4 IU/hr. The glucose infusion rate (GIR) was adjusted to maintain a steady-state blood glucose level of 100 mg/dl  $\pm$  10mg/dl. All intravenous infusions were administered and controlled using Alaris IVAC IV infusion pumps. Once this level held for 30 minutes, the dog was considered 'clamped' and the test article, in this case an iv insulin bolus was administered.

By accident, instead of 1.8 IU/kg insulin, a total dose of 180 IU was administered iv, a 100X overdose. The error was recognized immediately and the glucose infusion was aggressively increased and the insulin infusion was

discontinued. Blood chemistry values were measured using a commercial blood chemistry analyzer and a potassium infusion was added to bring the blood potassium values back to within normal parameters. The clamp was maintained until the dog was fed and capable of maintaining blood glucose of greater than 70 mg/dl without supplemental glucose. This was approximately 9 hours after the administration of the insulin bolus. The following day, blood chemistry was measured twice and serum samples were collected for insulin concentration determination.

### **7.2.2 Analytics**

The all blood samples for serum insulin and c-peptide measurements were allowed to clot for 45 minutes at room temp in BD Microtainer Serum Separation Tubes. The samples were spun at 5g for 5 minutes. The serum was collected into clean 1.5 ml Eppendorf centrifuge tubes and stored at  $-20^{\circ}\text{C}$  until assay. The serum insulin concentrations were determined using the Linco commercial ELISA EIA kit (EZHI-14K), Linco Research, St. Charles, Missouri.

## **7.3 Results and Discussion**

Due to aggressive management of the glucose clamp, the blood glucose never dropped below 50 mg/dl and never exceeded 300 mg/dl. Figure 7.1 presents the blood glucose, glucose infusion rates and blood insulin levels of the dog throughout the procedure. The peak insulin value exceeded 30 IU/liter briefly shortly after the insulin bolus. The serum insulin concentration remained above 10 IU/liter for more than 75 minutes and was higher than 1 IU/liter for more than three hours. During this

time, the dog displayed minor discomfort, yawning/gagging, as well as periods of tachycardia and shallow respirations. Mostly, the dog slept. The basal blood glucose concentration of the clamp was initially raised to greater than 250 mg/dl to prevent the blood sugar from plummeting and was gradually and painstakingly reduced over time to the pre-dose levels of approximately 75 mg/dl. The dog stood to stretch and urinate approximately every 90 minutes and showed interest in food approximately 7 hours post-dose. Once the dog was fed and appeared bright, alert and reactive, he was returned to her pen overnight. The dog recovered fully and returned to the protocol. Figure 7.2 shows the procedure repeated one week later with an oral insulin-loaded hydrogel dose. The dog showed normal and expected responses and a tight clamp was maintained. Table 7.1 shows the detailed blood chemistries obtained during the procedure. As the potassium levels were very low during the procedure, intravenous potassium was administered to maintain critical electrolyte balance.

#### **7.4 Conclusions**

The blood glucose and insulin levels that were drawn during the management of the insulin overdose, help explain the two sites of insulin saturability. The fact that the serum insulin concentration remained above 10 IU/liter for more than 75 minutes and was higher than 1 IU/liter for more than three hours, supports the view that there is a saturable process that transports the insulin from the plasma to the interstitial fluid.<sup>2</sup> Dernovsek found that insulin was not degraded by endothelial cells, but rather, it was able to pass through the cells.<sup>3</sup> Later, King showed that endothelial cells are able to transport insulin across them, most likely by a specific receptor-mediated

process.<sup>4</sup> A study looking at insulin concentrations in both the plasma and lymph fluid, found that the transport of insulin across the endothelial cells was mostly due to diffusion, and that an insignificant receptor-mediated component may also exist.<sup>5</sup> However, in our model, the insulin levels may have become so un-physiologic that the insignificant receptor-mediated component became significant, and the diffusion pathway may have been saturated. Dernovsek postulated that the endothelial cells could be used as a storage area for insulin, but our data can neither support nor counter this theory.<sup>3</sup>

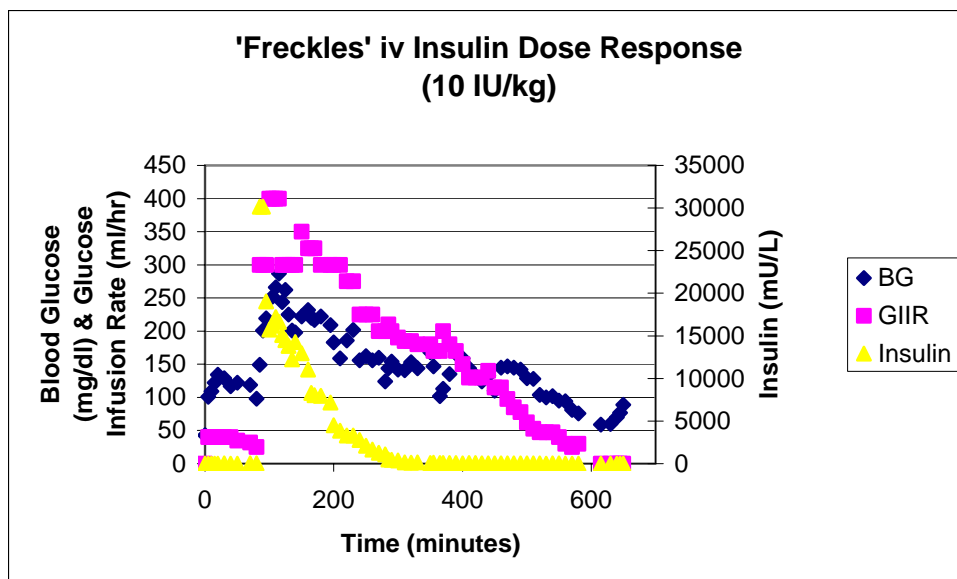
The amount of glucose that needed to be infused to maintain adequate blood glucose, even after the plasma insulin declined, supports the idea that there is a second site of insulin saturation.<sup>2</sup> This site of insulin saturability is less controversial, as much is known about the insulin receptor and its downstream effects. 60-80% of the insulin is taken up by the liver, 10-20% by the kidneys, and the remaining insulin is taken up by the skeletal muscle and adipose tissue. It has been thought that lymphocytes and adipose tissue may act as storage areas for insulin, since it was found that they both release non-degraded insulin.<sup>6,7</sup> Whether this mechanism played a role when the interstitial fluid was flooded with insulin is unknown.

The high levels of insulin in the plasma for an extended period of time, and the high levels of glucose that needed to be infused, even after the plasma insulin levels declined, support a two receptor site saturability.

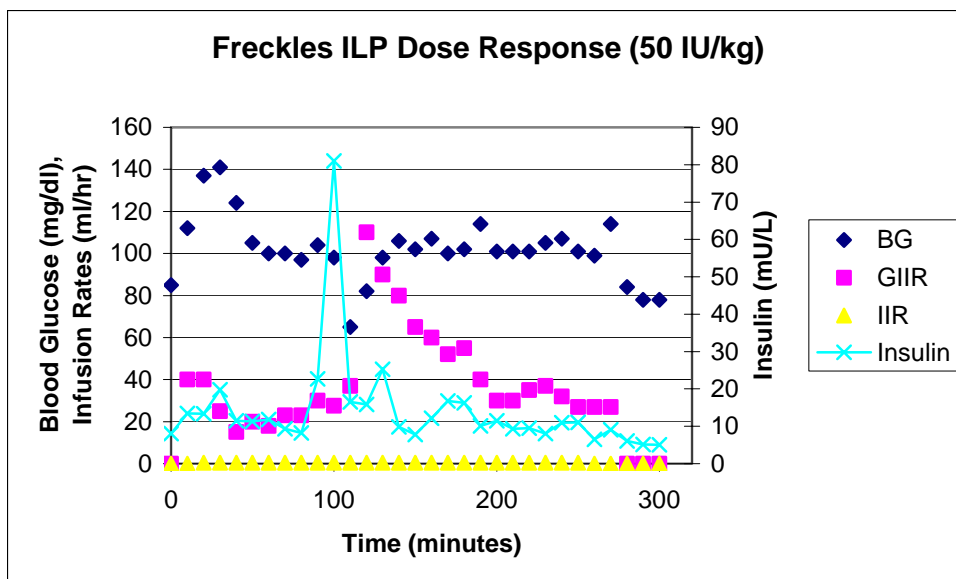


**Table 7.1: Canine Blood Chemistry Values from 10 IU/kg Dose**

<b>Time (min)</b>	<b>BG (mg/dl)</b>	<b>GIR (ml/min)</b>	<b>K<sup>+</sup>IR</b>	<b>BUN</b>	<b>HcT</b>	<b>Na<sup>+</sup></b>	<b>K<sup>+</sup></b>
190	186	280	14	7	24	137	2
240	156	225	14	7	21	135	2
310	141	185	16	7	26	139	2.2
405	160	140	16	6	25	139	2.9
470	147	97.5	16	5	26	141	3.5
515	106	47.5	12	5	29	142	3.7
545	102	47.5	10	4	26	148	3.3
585	78	30	10	4	25	142	3.3
640	70	0	0	4	30	145	3.1
1590	95	0	0	9	45	146	4.2
1820	100	0	0	15	47	145	4



**Figure 7.1: Blood Glucose and Serum Insulin during 10 IU/kg Dose**



**Figure 7.2: Oral Hydrogel Dose Response 1 week later on same dog as received the 10 IU/kg Dose**

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## CHAPTER 8: CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

### 8.1 Conclusions

The overall goal of this work was to provide the most detailed and realistic *in vitro* and *in vivo* analysis on the P(MAA-g-EG) hydrogel system to date and to establish a clear path forward for the optimization and, potentially, commercialization of this novel oral protein delivery vehicle. During this evaluation, a single formulation of the hydrogel was used consistently throughout the characterization to limit the introduction of extraneous variables that could complicate the analysis.

Hydrogel preparations consisted of a 1:1 monomer ratio of methacrylic acid to ethylene glycol diluted to a solvent fraction of 44.7% (wt./wt.) ethanol and water. The resulting hydrogel films were ground down into particles and sieved resulting in a maximum particle size of 43  $\mu\text{m}$ . The polymer was loaded with insulin to an average of 6% of total weight of the resulting ILP. The ILP batches were prepared for three campaigns; the cell work, the rat study and the canine study and the average insulin loading percentage from each batch was used for the entire campaign.

During the cell work, the insulin transport capability of the system was confirmed using the Caco-2 cell line. The ability of the hydrogel system to protect entrapped insulin from the chemical and biochemical hazards of the upper gastrointestinal tract was confirmed via dissolution studies in simulated gastric fluids containing active pepsin. Further the pH-responsive behavior of this system was confirmed via a serial dissolution study in simulated gastric and simulated intestinal fluids each containing active enzymes. The enhanced permeability of insulin in the Caco-2 cell line relative to insulin alone was confirmed via transport studies using

semi-permeable membranes. The correlation between cell permeability and transepithelial electrical resistance (TEER) was determined and it was found that the maximum cell permeability did not always correspond with the largest variations in TEER, suggesting that paracellular transport may not be the sole mechanism of transport enhancement of the polymer system.

The *in vivo* characterization of the system began with an iv-crossover-to-oral study in Sprague-Dawley rats. The absolute bioavailability in rats was determined for a single ILP dose, 50 IU/kg. The bioavailability obtained in this study was significantly less than that reported in prior rodent *in vivo* studies<sup>1</sup>. As the rodent model has significant limitations for absolute bioavailability studies such as limited blood sampling, risk, difficulty and complications of delivering the ILP via gavage or gelcap etc., the system was evaluated in a canine model. Hyperinsulinemic euglycemic insulin clamping was employed as a basis of an analogous iv-crossover study in healthy female dogs. The resulting absolute bioavailabilities determined were essentially equal to those found in the rat model.

Lastly, as the bioavailability values obtained in these *in vivo* studies were significantly lower than expected, pharmacokinetic models were employed to determine the nature and location of the drug loss. A hepatic cannulation model based upon the assumptions that drug metabolism/loss occurs in a series fashion based upon the site of administration suggests that most of the insulin is metabolized/lost at the gut wall.

## 8.2 Recommendations

The results of this work offer a clear path forward for optimizing this promising oral protein delivery system. If transport across the intestinal mucosa and gut wall can be improved, even by as little as 20%, there will be significant increases in oral bioavailability. For example, a 15% reduction in resistance at the gut wall can yield a six-fold increase in bioavailability as demonstrated in Figure 6.6. Further, as the ability of the system to protect entrapped proteins through the upper gastrointestinal tract and to release the cargo in the small intestine have been confirmed, this system could be very valuable as a vehicle to deliver drugs locally to the intestinal mucosa. The remainder of this chapter will propose several paths forward.

### 8.2.1 Optimization of Mucoadhesion

In the consideration of improving uptake of protein across the gut wall, two aspects should be considered; the creation and maintenance of a maximum concentration of drug on the apical surface of the mucosa as well as optimizing transport across the mucosa. As applied to the current work, the ILP will very quickly deliver the payload once it reaches an area of adequately elevated pH. Once released, the protein is mixed, and effectively diluted, with the chyme and other contents of the intestinal lumen. It would be ideal if the release could be more specific and localized. To this end, there is already ongoing work on mucoadhesion. This is not a new concept and, in fact, optimization of the mucoadhesive properties of this hydrogel system date back a decade and the current formulation is the result of

significant work on that front <sup>2</sup>. That said, there remains room for improvement. An ideal situation would be the case where the polymer particles attached well to the intestinal mucosa and drug release was one-directional towards the gut wall. This would eliminate or greatly reduce the amount of drug lost in dilution in the intestinal contents and/or destroyed by proteolytic enzymes of the small intestine. He et al reports the development of one such device in the recent literature <sup>3</sup>. This group has developed a self-folding miniature device comprised of a finger-like bilayer structure made up of pH-sensitive hydrogel layer and a non-swelling layer based on poly(hydroxyethyl methacrylate) (PHEMA). A mucoadhesive drug layer is attached on the bilayer and the authors propose that this self-folding device first attaches to the mucous and then curls into the mucous based upon the different swelling in the bilayer. It is also proposed that the PHEMA layer can serve as a diffusive barrier to prevent drug leakage into the intestine. This mico device concept has also been explored in approaches such as intestinal patches. An intestinal patch system for the delivery of erythropoietin (EPO) has recently been reported and has shown some capability to enhance EPO transport in the presence of absorption enhancers in *in situ* rat studies<sup>4</sup>.

### **8.2.2 Optimization of epithelial transport**

Once adequate and sustained contact with the epithelial membrane has been achieved, the ability of the delivered drug to cross the gut wall must be enhanced. This is truly a case of man versus nature as the gut wall is specifically designed and regulated to prevent the passage of pathogens and toxins, many of which are proteins.



As a mixed mechanism of insulin absorption cannot be ruled out based on the work to date, both paracellular and transcellular mechanisms could possibly be exploited. It has been theorized that the system has the ability to loosen tight junctions via the uptake of calcium ions and that this mechanism may play a key role in the increased insulin bioavailability seen in early *in situ* studies<sup>5,6</sup>. This may be true but this mechanism must be approached carefully as previous work using calcium chelators have led to unacceptable side effects<sup>7</sup>. A more optimal route would be to consider exploiting the zonulin system. The zonula occludens toxin (ZOT) is known to play a significant role in the modulation of tight junctions<sup>8</sup>. Alternatively, mechanisms to enhance and promote active transport across the epithelial cells by insulin receptors should and are being considered. Lastly, while there is significant effort and promise in these approaches, a far deeper understanding of cell biology is required and a collaboration that brings deep cell biology expertise onboard with the current program should be seriously considered.

### **8.2.3 Application of P(MAA-g-EG) system to other drugs**

Another opportunity for increasing the utility is to consider applying the system for targeted versus systemic therapies. By modifying the polymer to enable the delivery of other protein therapeutics, especially drugs targeting the gastrointestinal tract, the potential for commercialization may be greatly enhanced. One example would be to deliver antibody directly to the intestinal mucosa for applications such as Crohn's disease. Not only would this approach play to the current strengths of the P(MAA-g-EG) system, it would greatly reduce the need for

the drug to be systemically available, and, therefore, reduce the need to transport active protein and peptide therapeutics across the intestinal mucosa.

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